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(71) Applicant: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US).

(72) Inventors: CAPRIOLI, Richard, M. ; 37 East Brod Oaks, Houston, TX 77000 (US). PHOEBE, Charles, H., Jr. ; 9 My Way, Uxbridge, MA 01564 (US). JARRELL, Joseph, A. ; 11 High Street, Newton Highlands, MA 02161 (US).

(74) Agent: MAYFIELD, Denise, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

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(54) Title: DERIVATIZATION AND IDENTIFICATION OF SACCHARIDES

(57) Abstract

The present invention provides a method for the complete analysis of saccharides. The inventive method is directed to the production of derivatized saccharide compounds by reacting an organic dye molecule, Lucifer yellow CH (4-amino-N-[(hydrazinocarbonyl)amino]-2,3-naphthalimide-3,6-disulfonate, or related 4-aminonaphthalimide, to produce derivatized saccharide molecules which can be effectively analyzed using several known techniques.

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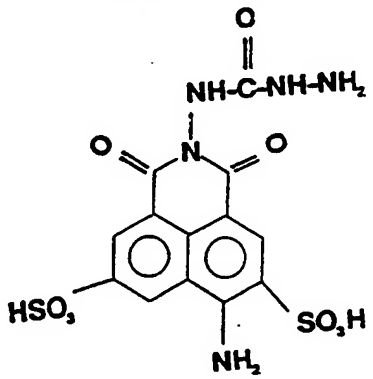
DERIVATIZATION AND IDENTIFICATION OF SACCHARIDES

The present invention is generally directed to the analysis of saccharides in vivo and in vitro, and more particularly, to the derivatization of saccharides using 4-aminonaphthalimide fluorescent dyes.

4-aminonaphthalimides are fluorescent dyes which are used to visualize living cells. The 4-aminonaphthalimides fluorescent dyes are generally nontoxic at concentrations and levels used for cellular illumination. Lucifer yellow CH is a 4-aminonaphthalimide fluorescent dye which was introduced in 1978 by the Aldrich Chemical Corporation, Milwaukee, Wisconsin. Chemically, Lucifer yellow CH (LyCH) is 4-amino-N-[(hydrazinocarbonyl)amino]-2,3-naphthalimide-3,6-disulfonic acid. The compound is commonly available and is used as the dipotassium or dilithium salt. The structure of Lucifer yellow CH is shown below:



Since its introduction in 1978, LyCH has been used with considerable success as a fluorescent intracellular marker in a wide variety of biological systems. The spectral properties of the dye include: absorption maxima at 280 nm and 430 nm, corrected emission maxima near 540 nm, and quantum yields of about 0.25. The high quantum yi lds make possible the detection of the dye at low concentration, and the wide separation between the absorption and emission maxima facilitates excitation at

one wavelength and observation of fluorescence at another.

LyCH has a free hydrazido group which reacts with aliphatic aldehydes at room temperature. In cellular research, aldehyde-containing fixatives are used to bind the dye to tissue, presumably through the hydrazido group. Even more specifically, Lucifer Yellow CH has been used in the fluorescent labeling of cell surface glycoconjugates and gangliosides.[2] (Spiegel *et al.*, (1983), *Biochem. Biophys. Res. Commun.*, *112*(3):872-77). The Lucifer Yellow CH compound has been used in carbohydrate work. Specifically, this class of dyes has been used as a carbohydrate stain to detect the presence of carbohydrates on the surface of cells. Spiegel *et al.* were simply determining whether or not cells had carbohydrates on their surfaces, and not attempting to quantitate or qualitate the presence of one or more saccharides in the test system. Aqueous solutions of this dye appear to be chemically stable for at least several months at room temperature.

A frequent application of LyCH has been to reveal the shape of individual neurones. It has been used to determine the branching pattern and course of regenerating neurones. Because of the low toxicity of the dye, direct *in vivo* observations on the regeneration of dye-filled neurones is possible. Other uses of LyCH dye include intracellular staining followed by immunocytochemical staining with rhodamine-labelled antibodies; and retinal staining in a calcium-free Ringer's solution.

The present invention presents a method for the derivatization of saccharides and mixtures of saccharides using an organic dye molecule, Lucifer Yellow CH, and

compounds related thereto. Saccharide derivatization using this and related dyes permits the use of a variety of qualitative and quantitative analytical procedures.

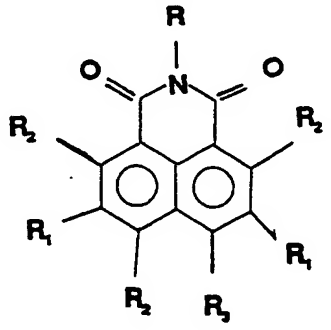
More particularly, the present invention is directed to the use of 4-aminonaphthalimide fluorescent dyes, including for example LyCH, in the analysis of saccharides. The use of this and related organic dye molecules has been found to amazingly enhance the ability to detect and quantify femtamole levels of any included saccharide. Even more particularly, the use of these intensely fluorescent compounds, which have previously been appreciated only as tracer tags in microscopic studies, are now used in the preparation of specific saccharide derivatives. Derivatization of saccharides according to the present invention has been found to alter both the physical and chemical properties of the saccharide molecule. The discovery of this particular effect on the saccharide molecule defines in part one point of novelty in the described invention. Related thereto, the use of the described methods impart a characteristic charge to the derivatized saccharide, which allow for the separation of the derivatized saccharides by electrophoretic techniques. Applicants have found this physical and chemical modification of the saccharide to make them particularly amenable to a variety of qualitative and quantitative analytical procedures.

The following description is presented to clearly define those compounds included within the definition of "saccharides". Saccharides are carbohydrate compounds. Carbohydrates are polyhydroxy aldehydes, polyhydroxy ketones, or compounds which can be hydrolyzed to form them. A carbohydrate that cannot be hydrolyzed to a simpler compound is a monosaccharide. A carbohydrate

which is hydrolyzed to two monosaccharides is a disaccharide. A carbohydrate which can be hydrolyzed into many saccharide molecules is called a polysaccharide or oligosaccharide. A monosaccharide can be further classified. If it contains an aldehyde group, it is known as a aldose; if it contains a ketone group it is known as a ketose. Carbohydrates which reduce Fehling's, or Tollens' reagent are referred to as reducing sugars.

Saccharides are an extremely important group of compounds in biological systems. Saccharides are one of the basic biochemicals in living systems. For example, saccharides alone, or in combination with other biological compounds, are utilized in living systems as energy sources, structural components, nerve impulse transmission, and the immunological response mechanism. Because of the importance of these compounds, a method to identify, analyze, and isolate select saccharides would be very advantageous. Accordingly, the present invention is directed to providing a method for preparing saccharide derivatives which are easily identified, analyzed, and isolated.

One aspect of the present invention is directed to a method for preparing a derivatized saccharide. In one preferred embodiment of the inventive method for the derivatization of a saccharide, the saccharide is reacted with a compound having the formula:



and salts thereof, where R is $NHCONHNH_2$ or $CH_2(CH_2)_n NH_2$ or other chemical group that can covalently interact with the reducing end of a saccharide; and where R_1 is CO_2^-, OPO_3^- or SO_3^-, or any other group which would impart a charge or water solubility to the molecule; and where R_2 is a hydrogen, halide or a methyl group, and where R_3 is NH_2 or H. More particularly, R may be any other group that interacts with the (C=O) group of a saccharide. Where R is $CH_2(CH_2)_n NH_2$, it is expected that the double $-(CH_2)_n-N=C-$ Schiff base formed should first be reduced with for example, sodium borohydride, to form a more stable bond, such as, for example, $-(CH_2)_n-N_H-CH_2-$. When R is $CH_2(CH_2)_n NH_2$, "n" is further defined as 2, 3 or 4, and refers to the number of CH_2 groups forming the internal carbon chain of the R group in the defined compound.

In a most preferred embodiment of the above described molecule, R is $NHCONHNH_2$, R_1 is SO_3^-, R_2 is H and R_3 is NH_2. It is to be understood, however, that other substitutions can be made in the above-described molecule with retained functionality of the molecule for use in the described inventive methods of saccharide derivatization, separation and identification.

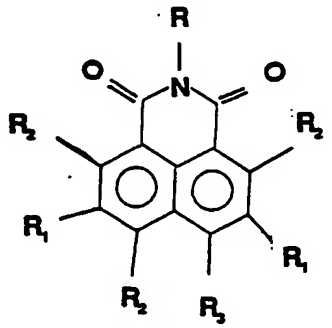
In accordance with one embodiment of the invention, the method also includes the additional step of separating the derivatized saccharide. Preferable techniques for separating the derivatized saccharide include, by way of example, and not limitation, gas chromatography, liquid chromatography (HPLC), paper chromatography, thin layer chromatography, and electrophoresis.

In accordance with a further embodiment of the invention, the method includes the additional step of identifying a saccharide. The identification of

saccharides may be performed on mixtures of monosaccharides as well as mixtures of polysaccharides. Preferable techniques for identifying the saccharide include by way of example and not limitation, HPLC, NMR, fluorescence spectroscopy, and mass spectrometry.

The saccharide which is derivatized may be a monosaccharide, disaccharide, polysaccharide or oligosaccharide. According to another preferred embodiment of the invention, the saccharide derivatized comprises an amino-saccharide or a N-acetylated saccharide.

Another preferred embodiment of the invention comprises a method for the identification of saccharides of a glycoprotein. In a preferred embodiment of the claimed method for identifying the saccharides of a glycoprotein, the method comprises the steps of: separating the saccharides from the glycoproteins; mixing the saccharides in the presence of an acid with a compound having the following structure:



or salts thereof, wherein R is $CH_2(CH_2)_nNH_2$ or $NHCONHNH_2$, R_1 is SO_3^-, CO_2 or OPO_3^-, R_2 is hydrogen, halide or methyl, and R_3 is NH_2 or H, to form a reaction mixture; heating th reaction mixture for betw en 30 minutes to 180 minutes to form a mixture of derivatized saccharides; separating the

mixture of derivatized saccharides using one or more of gas chromatography, liquid chromatography, paper chromatography, thin layer chromatography, and electrophoresis; and identifying the saccharides of the glycoprotein using HPLC, NMR, fluorescence spectroscopy or mass spectrometry. Most preferably, the reaction mixture is heated for about 90 minutes (1.5 hours).

In a most preferred embodiment of the claimed method, the compound is further more closely defined wherein R is $NHCONHNH_2$, R_1 is SO_3^-, R_2 is H, and R_3 is NH_2. This compound is Lucifer Yellow CH. When R is $CH_2(CH_2)_n NH_2$, "n" is further defined as 2, 3 or 4, and refers to the number of CH_2 groups forming the internal carbon chain of the R group in the defined compound.

In one preferred aspect of the claimed method, the saccharides are a mixture of monosaccharides and the reaction mixture is to be heated for about 90 minutes. The separation of the saccharides from the glycoprotein may be accomplished through the use of a variety of techniques well known to those of skill in the art. By way of example, the saccharides may be separated from the glycoprotein through hydrolysis of the glycoprotein to form a mixture of saccharides. The mixture of derivatized saccharides is most preferably separated by electrophoresis, with the saccharides being identified most preferably by the process of HPLC.

The glycoprotein is most preferably reacted with the defined compound in the presence of acetic acid.

The following abbreviations are used throughout the specification:

mV = millivolts

Man	=	mannose
LC	=	liquid chromatography
mw	=	molecular weight
M	=	molar
V%	=	volume percent of the solution
μl	=	microliter
μA	=	microangstrom
Glc	=	glucose
Gal	=	galactose
TFA	=	trifluoracetic acid
GalNAC	=	N-acetylgalactosamine
GlcNAc	=	N-acetylglucosamine
Fuc	=	fucose
NAN	=	N-acetylneuraminate (sialic acid)
CZE	=	capillary zone electrophoresis

FIG. 1 is a standard curve for derivatization and chromatography of neutral and N-acetylated monosaccharides: closed circle = glucose; downward pointing open triangle = galactose; upward pointing open triangle = mannose; open circle = fucose; X = N-acetyl glucosamine; and open square = N-acetylated galactosamine.

FIG. 2 is a standard curve for derivatization and chromatography of glucosamine and galactosamine: open square = glucosamine, 1.5 hour reaction; X = glucosamine, 1.0 hour reaction; open triangle = galactosamine, 1.0 hour reaction, earlier peak; closed triangle = galactosamine, 1.0 hour reaction, later peak; and open circle = galactosamine, 1.0 hour reaction summation of both peaks.

FIG. 3 is a standard curve for derivatization and chromatography of N-acetyl neuraminic acid showing four distinct chromatographic peaks.

FIG. 4 is a standard curve for derivatization and chromatography of neutral and N-acetylated monosaccharides: closed circle = glucose; downward pointing open triangle = galactose; upward pointing open triangle = mannose; open circle = fucose; X = N-acetyl glucosamine; and open square = N-acetylated galactosamine.

FIG. 5 is a standard curve for derivatization and chromatography of glucosamine and galactosamine: open square = glucosamine, 1.5 hour reaction; X = glucosamine, 1 hour reaction; upward pointing open triangle = galactosamine 1 hour reaction.

FIG. 6 is a standard curve for derivatization and chromatography of N-acetyl neuraminic acid.

FIG. 7 is a standard curve for derivatization and chromatography of galactose; fluorescence detection: excitation 430 nm; emission 500 nm.

FIG. 8 is a standard curve for derivatization and chromatography of a mixture of monosaccharides.

FIG. 9 is a standard curve for derivatization and chromatography of individual monosaccharides in a mixture.

FIG. 10 - HPLC Analysis - Fluorescence detection of Lucifer Yellow CH-derivatized mixtures of monosaccharides.

FIG. 11 - Fluorescence detection of Lucifer Yellow CH-derivatized mixtures of monosaccharides. The range expansion in the second plot was by comput r; 11A - HPLC = 10 μl of a mixture of monosaccharides, each at 0.001 M

(in 0.2 M acetic acid) derivatized with 10 μl of a 0.1 M Lucifer Yellow CH for 1.5 hours at 72°C; 11B - acetic acid derivatized with 10 μl 0.1 M Lucifer Yellow CH for 1 hour at 72°C 430 mm Ex, 500 nm EM cut-off filter, 1 μA range setting. HPLC - Control reaction 10 μl of 0.2 M.

FIG. 12 - Column Retention time for Lucifer Yellow CH. The figure demonstrates the gradual loss in Lucifer Yellow CH from a packed Nova-pac 18 column over a period of about 20 days.

FIG. 13 - Hydrolyzation of Dextran to a mixture of oligosaccharides. Dextran was hydrolyzed to form a mixture of different oligosaccharides. These oligosaccharides were then derivatized with Lucifer Yellow; 13A - A_{210}; 13B - Fluorescence, Ex 430 nM; EM 500 nm, cut-off filter, 1 μA Range (least sensitive).

FIG. 14 - Mass Spec Analysis - Separation of LyCH-polysaccharide mixtures.

FIG. 15 - Mass Spec Analysis - Electrophoretic analysis of LyCH monosaccharides.

FIG. 16 - Mass Spec Analysis - LC separation of LyCH monosaccharides after storage; 16A - LyCH monosaccharides at 0 min. storage; 16B - LyCH monosaccharides after overnight storage at <-10°C; 16C - LyCH monosaccharides after overnight storage at 0-5°C.

FIG. 17 - Mass Spec Analysis - Fast atom bombardment mass spectrometric analysis (Negative Ion) using Marks Glycerol Matrix ions; 17A - Fucose - LyCH; 17B - Glucosamine - LyCH; 17C - Glucose - LyCH; 17D - N-acetyl glucosamine - LyCH.

FIG. 18 - Mass Spec Analysis - Microbore liquid Chromatography/mass Spec Analysis of Glc-Lucifer Yellow CH (18-A) and Maltotetraose - Lucifer Yellow CH (18-B).

FIG. 19 - Mass Spec Analysis - Maltotetraose - LyCH alone and with one or two ion pairing reagents.

FIG. 20 - Mass Spec Analysis - Glucose LyCH with one or two molecules of an ion-pairing reagent.

FIG. 21 - HPLC Analysis - Effect of Reaction Time (21A) and Reaction Volume (21B) on Derivatization of a mixture of monosaccharides with Lucifer Yellow CH monosaccharides at 0.0025 M in the reaction.

21A - 1 = 1 hour Reaction Time
21A - 2 = 1.5 hours Reaction Time
21A - 3 = 2 hours Reaction Time
21B - 1 = 1 x (20 μl sample)
21B - 2 = 2 x (40 μl sample)
21B - 3 = 5 x (100 μl sample)
21B - 4 = 1 x (Control)
Reactions were heated for 2 hours at 72°C.

FIG. 22 - HPLC Analysis - Derivatization of Galactosamine with Lucifer Yellow CH-Standard Curve, A_{210}, Expanded.

FIG. 23 - Proton NMR Spectrum for Lucifer Yellow CH-Glucose Reaction Product.

FIG. 24 - Major Derivative Fragmentation Pathway Suggested by MS/MS Analysis.

The present invention provides a method for the identification of saccharides. The inventive method also

relates to the production of derivatized saccharide compounds. More particularly, the present method is directed to the use of an organic dye, Lucifer yellow CH (acid or salt form of 4-amino-N-[(hydrazinocarbonyl)amino]-2,3-naphthalimide-3,6-disulfonate) and related 4-aminonaphthalimide compounds in conjunction with a variety of derivatized saccharides. The use of these particular dye compounds in conjunction with the derivatized saccharides have been found to provide surprisingly enhanced methods of identifying individual saccharides.

It has been discovered that LyCH and related compounds bind to the reducing end of a saccharide molecule. As used in the present application, the reducing end of a saccharide is defined as the aldehyde end of a saccharide molecule. More specifically, it is believed that LyCH, through its free hydrazido group, binds to the carbonyl at the reducing aldehyde end of the saccharide molecule. Therefore, other related 4-aminonaphthalimides compounds which include similar functional groups, such as carboxyls or phosphates in place of the SO_3H, are included with the scope of this invention. Any R group which covalently binds to the reducing end (carboxyl) of a saccharide may be used as part of the described molecule in conjunction with the present invention.

The derivatization of the saccharide molecule alters its physical and chemical properties, making it amenable to qualitative and quantitative analytical procedures. Applicants have employed the intense fluorescence of the particularly defined 4-aminonaphthalimide compounds in the surprisingly sensitive detection and separation of derivatized saccharides at femtomole levels. Another feature of the described methods includes the ability to

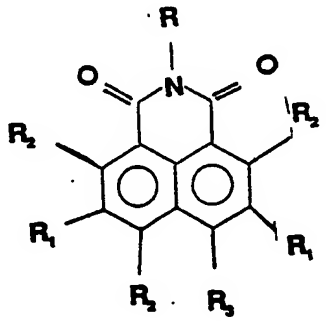
use a variety of analytical methods in the separation, detection, and identification of the derivatized saccharide. By way of example, methods for identification and separation found amenable with the described modified saccharides include, liquid and thin layer chromatography, gas chromatography, paper chromatography, electrophoresis, nuclear magnetic resonance spectrometry, HPLC, fluorescence spectroscopy, and mass spectrometry. Such is possible through the unique specific chemical and structurally changed derivatized saccharides described in the inventive methods outlined herein. It is should be noted that one or more specific analytical techniques can be used to both separate and identify the derivatized saccharide, for example electrophoresis, liquid and thin layer chromatography. Electrophoretic separation of the derivatized saccharides is possible through the imparting of a charge to the derivatized saccharides. Such is not typically possible with sugars, as these substances are not charged molecules.

The present invention has determined that LyCH is useful in the derivatization of reducing carbohydrates. The detection limits of LyCH-derivatized saccharide with a 210 nm absorption are within an order of magnitude of that for the dabsylhydrazine derivative (UV at 425 nm, 10 pmole lower limit, Lin and Wu, *Analytical Chemistry*, *59*:1320-1326 (1987)). Accordingly, fluorescence detection lowers the detection limit for the LyCH-saccharide derivatives.

The present invention demonstrates, in the following examples, the derivatization of several monosaccharides. Disaccharide, polysaccharide and oligosaccharide molecules have been similarly derivatized. Results of work with oligosaccharides have demonstrated that

chemical and analytical procedures similar or identical to those used for monosaccharides are also effective with oligosaccharides.

According to one preferred embodiment of the invention, a method is provided for preparing a derivatized saccharide. In accordance with the invention, the saccharide is reacted with a compound having the formula:



or salts thereof, wherein R is $CH_2(CH_2)_n NH_2$, NH-CO-NH-NH_2, or other chemical group that can covalently interact with the reducing end of a saccharide. In the above molecule, R_1 is CO_2^-, OPO_3^- or SO_3^- or other group which would impart a charge or water solubility to the molecule. R_2 is hydrogen, a halide or a methyl group, and R_3 is NH_2 or H. When R is $CH_2(CH_2)_n NH_2$, "n" is further defined as 2, 3 or 4, and refers to the number of CH_2 groups forming the internal carbon chain of the R group in the defined compound.

Most preferably, R is -NH-CO-NH-NH_2, R_1 is SO_3^-, R_2 is H and R_3 is NH_2, and the compound reacted with the saccharide is LyCH. Where R compris s a functi nality which may function as a Schiff base, the compound should most preferably be reduced with a reducing agent (i.e.,

so as to comprise an N=C group where R= $(CH_2)_n(NH_2)$, it is expected that the double N=C bond of the terminal amino group most preferably should first be reduced. For example, the reducing agent, sodium borohydride, may be conveniently used to reduce a Schiff base to form a more stable secondary amine.

In a most preferred embodiment of the described molecule, R is NH-C-O-NH-NH_2, R_1 is SO_3^-, R_2 is hydrogen, and R_3 is NH_2. It is to be understood, however, that other substitutions can be made in the above described molecule with retained functionality of the molecule for use in the described inventive methods of saccharide derivatization and their separation and detection.

In accordance with one embodiment of the invention, the reaction procedure consists of adding a sufficient amount of LyCH to a quantity of one or several monosaccharides in an acidic solution. The final concentration of monosaccharide in the reaction preferably ranges from about 0.0000125 M to 0.5 M. The reaction is preferably heated at a temperature of from about 25°C to about 100°C, and more preferably at about 72°C.

The reaction is heated for from about 30 minutes to about 180 minutes, and most preferably for about 90 minutes, or one and one-half hours. It has been determined that the length of time the reaction is allowed to proceed affects how far the reaction moves toward completion. For example, it has been determined that the most preferred reaction time for the derivatization of a monosaccharide mixture appeared to be about 1.5 hours (or 90 minutes). Shorter reaction times have been found to improve the yield of certain LyCH-derivatized saccharides, for example NANA-LyCH. Longer

reaction times have improved the yield of the LyCH-derivatives of the amino sugars. At the end of the reaction period the reaction is stopped by sufficiently cooling the solution.

In accordance with one embodiment of the invention, the method also includes the additional step of separating the derivatized saccharide. Techniques for separating the derivatized saccharide include gas chromatography, liquid chromatography, paper chromatography, thin layer chromatography, and electrophoresis. High performance liquid chromatography (HPLC) is preferably used for the separation of the solvent and the derivatized saccharides. HPLC may be used for the simultaneous separation and identification of a derivatized saccharide.

Saccharides are separated by their characteristic elution time from an HPLC column. Further, these elution times are useful to characterize and identify particular saccharides. If HPLC analysis of the samples is to be carried out to either separate or identify derivatized saccharide molecules in the solution, the solution may be diluted with additional solvent.

In accordance with a further embodiment of the invention, the method includes the additional step of identifying the saccharide. Preferable techniques for identifying the saccharide include NMR, fluorescence spectroscopy, HPLC, and mass spectrometry. The most preferred method of identifying the derivatized saccharide is HPLC. HPLC chromatographies may be developed and compared with standards to precisely identify a particular derivatized saccharide. The inventors have also successfully used mass spectrometry

to identify particular derivatized saccharides (See Figs. 17, 19, 20 and Appendix 1).

The saccharide which is derivatized may be a neutral monosaccharide, disaccharide, polysaccharide, or oligosaccharide. According to one embodiment of the invention, the saccharide derivatized is a an N-acetylated saccharide. According to a further embodiment, the saccharide is an amino-saccharide.

The inventive method is useful for analyzing and identifying saccharide compounds *in vitro* and *in vivo*. The fluorescent properties of LyCH and related 4-aminonaphthalimides compounds makes possible the analysis of saccharides at lower levels than possible before. Further, the chemical and physical properties of these derivatized saccharides allows the analysis of saccharides using techniques such as gas chromatography, liquid chromatography, paper chromatography, thin layer chromatography, and electrophoresis which have not been as readily usable before.

The following Examples are presented to describe preferred embodiments and utilities of the present invention and are not to limit the present invention unless otherwise stated in the claims appended hereto.

EXAMPLES

Example 1
Derivatization of Monosaccharides

The following experiment was conducted to establish a standard procedure for the derivatization of various monosaccharides.

The reactivity of various monosaccharides with LyCH was compared using standard curves prepared for the sugars over a wide concentration range. All of the monosaccharides tested were obtained from Sigma Chemical Company, and were used without further purification. The LyCH was obtained from Aldrich Chemical Company. The same lot of LyCH was used for all of these studies. Monosaccharide stock solutions were prepared at 0.4 M in water and stored frozen. Dilutions of the stock sugar solutions were prepared in 0.2 M acetic acid just prior to derivatization. LyCH was prepared as a 0.1 M stock solution in water and stored frozen.

The reaction procedure consisted of adding 10 μL of a 0.1 M LyCH solution to 10 μL of monosaccharide solution in a glass dram vial with a Teflon-lined screw cap. The final concentration of monosaccharide in the reaction ranged from 0.000125 M to 0.05 M. The reaction was then heated at 72°C for one hour in a heating block. At the end of the reaction period, the vial was chilled on ice, and centrifuged briefly to pull the solvent down into the vial. The sample was then diluted to 0.5 mL with chilled water and stored at 0-5°C. High pressure liquid chromatography (HPLC) analysis of the samples was carried out within 30 hours of preparation.

It should be noted that during the heating of the reaction sample, the solvent evaporates and condenses inside the cap of the vial. It is believed that this concentrates the sugar and LyCH and drives the reaction in a forward direction. The presence of acetic acid at 0.1 M in the reaction appears to facilitate the solvent evaporation. In one experiment, three times as much LyCH-Glucose product was obtained when 0.1 M acetic acid was present in the reaction compared with the reaction in

water. Additional acetic acid did not increase the product yield.

The dilution of the final reaction with water is believed necessary to reduce the concentration of unreacted LyCH to 20 nmole/10 μL (maximal). It is believed that greater concentrations of LyCH may exceed the ion-pairing capacity of the HPLC mobile phase. Thus, this dilution of the final sample may impose a lower limit on the sugar sample concentration of about 0.25 mM if derivative detection is by UV absorption at 210 nm.

The derivatized monosaccharide samples were analyzed on a Nova-pak C18 (0.39 x 15 cm) stainless steel column kept at 50°C. The mobile phase solutions "A" and "B", respectively, were 0.005 M (aq) tetrabutylammonium phosphate (Low-UV PIC A) and Low-UV PIC A containing 40% (V%) acetonitrile. The flow rate was 1 ml/min. The solvent program was as follows: 15-35% B over 10 min; 35% for 5 min; linear reverse gradient to initial conditions over 5 min. This program was used for all standard curve analyses except those for the glucose derivative. The 100% B segment was omitted for the analysis of the glucose samples.

The results of the analysis of the individually derivatized monosaccharides are summarized in chromographs and in the standard curve plots shown in Figs. 1-9. These figures are standard curve plots obtained through the chromatographic separation and identification of the derivatized saccharides. Figs. 1-7 show that the reaction of LyCH with all of the sugars tested was linear over the concentration range shown with detection by UV absorption at 210 nm. Contaminating peaks in the LyCH made quantitation somewhat more difficult at the lower concentrations.

Table 1

Standard Curve Regression Parameters

For Injection of 0-1000 pmole, 1-Hr Reactions, Abs 210 nm

Monosaccharide	Correlation Coefficient	Slope[1]	y- Intercept[2]
Glucose	0.9990	1311	135505
Galactose	0.9932	1081	27128
Mannose	0.9976	1184	-5239
Fucose	0.9974	1272	64797
N-Acetyl Glucosamine	0.9975	1175	244907
N-Acetyl - Galactosamine	0.9994	1100	239320
Glucosamine	0.9977	283	18515
" (11.3 min)	0.9959	201	681
" (12.0 min)	0.9439	310	38237
Galactosamine sum of - 2 peaks	0.9710	511	38918
N-Acetyl Neuraminic Acid			
22.3 min	0.9971	196	-6667
22.7 min	0.9957	183	-9005
23.1 min	0.9968	281	-7991
25 min	0.9800	545	-27161
For 0-1000 pmole Injected, 1.0-Hr Reaction, Fluorescence			
Galactose	0.9964	47795	369403
For 0-1000 pmole Injected, 1.5-Hr Reaction, Abs 210 nm			
Glucosamine	0.9995	899	-17045

[1]Slope: (μV x Sec)/pmole

[2]y-Intercept: μV x Sec

With reference to Fig. 1, the slope of the standard curve for each of the neutral sugars, N-acetyl glucosamine (GlcNAc), and N-acetyl galactosamine (GalNAc), was essentially the same. On the basis of the amount of LyCH remaining in reactions containing 0.025 M monosaccharide (10 nmole monosaccharide injected, LyCH/sugar ratio 2/1), the reactions with the neutral sugars were found to have proceeded to 85% or greater

completion. Reaction yield for GlcNAc was 74% and for GalNAc was 56%. This suggests that the absorption at 210 nm by the N-acetylated sugars contributed to the observed response.

As shown in Figs. 2, 3, and 6, two of the sugars, galactosamine ($GalNH_2$) and N-acetyl neuraminic acid (NANA), gave more than one reaction product. The purity of the sugars was not assessed before derivatization and it is believed that more than one sugar may have been present in the original samples. Alternatively, the derivatization procedure may have produced more than the expected product with these sugars.

As seen in Fig. 2, glucosamine and galactosamine proved less reactive than the neutral sugars. The derivatization of glucosamine proceeded to only 40% completion by the end of the one-hour reaction time. Fig. 5 shows that increasing the reaction time to 1.5 hours, however, pushed the glucosamine reaction nearly to completion and resulted in a standard curve with a slope equivalent to that obtained with the neutral sugars. Results for galactosamine were less clear owing to the presence of two reaction products. On the basis of peak retention times, it is conceivable that the galactosamine sample contained a fair amount of glucosamine.

Fig. 4 shows that there were four fluorescent product peaks found for the NANA derivatized sample. The lowest concentration at which significant peaks above background were detected at 210 nm was 1 mM. This may be accounted for by impurities in the reagents that were used.

Fig. 7 shows the fluorescence detection of the galactose-LyCH derivative, using the least sensitive range setting of the Schoeffel FS 970 detector, which was 48 times more sensitive than detection by UV absorption at 210 nm. The presence of interfering LyCH contaminant peaks made fluorescence detection impractical for sugar solutions at concentrations less than 0.25 mM. This corresponds to a final sample load of 50 pmole on column.

Example 2
Mass Spectrometric Analysis of
Lucifer Yellow CH Monosaccharides Derivatives

Mass spectrometry was used to confirm the reaction of LyCH with the various monosaccharides. Analyses were carried out on a Finnegan MAT90 mass spectrometer operated in the negative ion FAB mode. Samples were introduced via the continuous flow probe with a solvent flow rate of 7 μL/min. The flow probe solvent was 5% (wt%) glycerol and 3% (V%) acetonitrile in water. One-μL samples were injected. The monosaccharide derivatives used were those prepared for the studies described in Experimental Example 1.

The mass spectra obtained for each of the LyCH-derivatized monosaccharides, with the exception of that for NANA-LyCH, all showed the presence of the expected $(M-1)^-$ ion for the derivative. The expected $(M-1)^-$ ion for the NANA-LyCH derivative is 735. This ion is also present in the glycerol background (See Appendix 1 - Glycerol Background, Negative Ion Mass Spec). Furthermore, the NANA-LyCH sample has shown the presence of at least four derivatives by HPLC and sample stability was uncertain. Nevertheless, with the exception of NANA-LyCH, each derivatized monosaccharide was positively identified, confirming the reaction of LyCH and the

saccharides (See Appendix 2 - MS Analysis of LyCH-derivatized saccharides).

Example 3
Derivatization of Mixtures of Monosaccharides

Various equimolar mixtures of the previously individually tested monosaccharides were derivatized simultaneously. The reaction procedure consisted of adding 10 μL 0.1 M LyCH to 10 μL of a mixture of monosaccharides in solution to a glass dram vial with a Teflon-lined screw cap. The mixtures of monosaccharides contained 0.001 M, 0.0025 M, or 0.005 M each of the sugars: galactosamine, glucosamine, mannose, galactose, glucose, N-acetyl galactosamine, N-acetyl glucosamine, fucose, and N-acetyl neuraminic acid. Most preferably, the reaction was at 72°C for 1.5 hours. The samples were then chilled on ice, centrifuged briefly, and finally diluted to 0.5 ml with water. A control reaction (containing no sugar) that was heated for 2 hours is also shown.

Reaction Conditions:

10 μl of a 0.1 M Lucifer Yellow CH (aq) solution was added to 10 μl of a mixture of monosaccharides in 0.2 M acetic acid. The mixtures of monosaccharides contained 0.001 M, 0.0025 M, or 0.005 M of each of the following sugars: galactosamine, glucosamine, mannose, galactose, glucose, N-acetyl galactosamine, N-acetyl glucosamine, fucose, and N-acetyl neuraminic acid. Reaction was at 72°C for 1.5 hours. The samples were then chilled on ice, centrifuged briefly, and finally diluted to 0.5 ml with water. A control reaction (containing no sugar) that was heated for two hours is also shown. High

performance liquid chromatography (HPLC) analysis of the samples was carried out within 30 hours of preparation.

HPLC Conditions:

Nova-pak C_{18} (0.39 x 15 cm) at 50°C.
A_{210}, 10 μl injection volume, 1 ml/min flow rate.
A: Low UV PICA (0.005 M tetrabutylammonium phosphate, aq)
B: 40% CH_3 CN_{TN} Low UV PICA.
Program: 10 - 100% soln. B over 30 minutes by curve 8
100% soln. B for 5 minutes
100% - 10% B linear gradient over 5 minutes.

Results for the HPLC analysis are shown in Fig. 8 for equimolar mixtures of the monosaccharides previously tested individually. Fig. 8 shows that the reaction was essentially linear over the concentration range shown for most of the sugars. Further, Fig. 8 shows that all the saccharides in the mixture were derivatized by the addition of LyCH.

Fig. 9 illustrates that the optimum reaction time for derivatization of the monosaccharide mixtures appeared to be about 1.5 hours or about 90 minutes. Shorter reaction times improved the yield of the NANA-LyCH derivatives while longer reaction times improved the yield of the LyCH derivatives of the amino sugars.

The effect of reaction time on derivatization of a mixture of monosaccharides and the effect of reaction volume on derivatization of a mixture of monosaccharides is presented in the HPLC analysis included herewith at Figs. 21A and 21B, respectively.

The effect of reaction volume scale-up is demonstrated at Fig. 21B. Monosaccharides at 0.0025 M were used in the reactions. Each of the reactions were heated for 2 hours at 72°C. The evaporation of reaction solvent is less complete for reactions scaled up two to five-fold (See Fig. 21B-2 and 21B-3). In the study, the reaction volumes of samples were 20 μl (1 x), 40 μl (2 x), and 100 μl (5 x). It was expected that larger volumes would diminish the amount of product obtained. Increasing the reaction volume had the greatest negative effect on the derivatization of the amino sugars, reducing yields by about 50%, while the yield for the NANA-LyCH peaks was unaffected. Neutral and N-acetylated sugars fared somewhere in between these two extremes.

Fluorescence detection of the Lucifer Yellow CH-derivatized mixtures of monosaccharides was also performed. Results are presented in the HPLC included herewith at Fig. 10.

Example 4
Characterization of LyCH Saccharides
By NMR Mass Spectrometric and HPLC Analysis

The following example was conducted to demonstrate the variety of quantitative and qualitative techniques which could be used in the analysis of the individual saccharides which typically comprise a glycoprotein. The particular saccharide derivatives analyzed in the present experiment include: Lucifer Yellow $CH(H_2)$ - Glucose; Galactose -Lucifer Yellow $CH(H_2)$; Fucose - Lucifer Yellow $CH(H_2)$; N-acetyl glucosamine-Lucifer Yellow $CH(H_2)$; Glucosamine-Lucifer Yellow $CH(H_2)$; Galactosamine Lucifer

Yellow CH(H_2); N-acetyl Neuraminic Acid-Lucifer Yellow CH(H_2); LyCH-Glucose; LyCH Maltohexaose; LyCH Maltotriose.

Saccharides were derivatized according to the methods described *infra*. Results obtained from this study demonstrate that the analysis of saccharides through the use of Lucifer Yellow organic dye saccharide derivatives is amenable to NMR spectrum analysis, HPLC analysis, and Mass Spectrum analysis with accurate and reproducible results.

Appendix 1 includes the mass spectrum analysis graphs of mannose-Lucifer Yellow CH(H_2); Glucose Lucifer Yellow CH(H_2); Galactose - Lucifer Yellow CH(H_2); Fucose - Lucifer Yellow CH(H_2); N-acetyl glucosamine-Lucifer Yellow CH(H_2); Glucosamine-Lucifer Yellow CH(H_2); Galactosamine Lucifer Yellow CH(H_2); N-acetyl Neuraminic Acid-Lucifer Yellow CH(H_2); LyCH-Glucose; LyCH Maltohexaose; LyCH Maltotriose; as well as a glycerol background.

Appendix 2 also included HPLC data from the Lucifer Yellow CH- derivatization of glucose, galactose, mannose, fucose, N-acetyl glucosamine, n-acetyl galactosamine, n-acetyl neuraminic acid (standard and expanded scale), and glucosamine (standard and expanded scale). The HPLC data from the Lucifer Yellow CH-derivatization of these saccharides is also summarized at Tables 3 and 4.

Appendix 2 includes HPLC analysis of the various forms of the derivatized saccharides. Results demonstrated in graphic form in Appendix 2 include analysis of the following saccharides: GalNH_2; GlcNH_2; Gal+Man; Glc; GalNAC; GlcNAc; Fucose; and NANA. Additionally, Appendix 2 includes the HPLC graph of LyCH-glucose, LyCH Mannose and LyCH glucose. The

proton NMR Spectrum for Lucifer Yellow CH-Glucose Reaction product is also included herewith in Fig. 23.

A presentation of the major derivative fragmentation pathway suggested by the mass spectrometric (single and double stage [MS/MS]) analysis is also presented at Fig. 24.

Example 5
Derivatization of Polysaccharides

The present experiment was performed to demonstrate the application of the described invention in the derivatization and separation of oligomers (as has already been demonstrated for monosaccharides). Dextran was hydrolyzed to a mixture of oligosaccharides, and even more particularly, malto oligosaccharides. Dextran was selected as the source saccharide in the present example. However, other sources of oligosaccharides may similarly be used in the practice of the described invention.

Oligomers obtained from the hydrolyzation of dextran were derivatized according to the following sequence of steps:

1. React 10 μl of a 0.1 M Dextran (average MW 9400) solution with 10 μl of a 0.1 M Lucifer Yellow for 1 hour at 72°C.
2. Dissolve Dextran in 0.2 M acetic acid.
3. Chill.
4. Centrifuge.
5. Dilute sample to 0.5 mL with low UV PIC A containing 6% CH_3CN (V%).
6. Centrifuge sample.
7. Chromatograph 10 μl of the soluble portion f the sample.

8. Perform HPLC on portion of the derivatized oligomer sample as described for derivatized mixtures of monosaccharides.

HPLC with the hydrolyzed Dextran mixture of oligosaccharides was performed according to the procedure described above. Results are presented in Figure 13. The HPLC also demonstrates the presence of a series of peaks, which represent the presence of a distribution of malto oligosaccharides in the test sample.

Example 6
Nova-pac C18 Column Preparation and Characterization

A Nova-pac C18 column (0.39 x 15 cm) is employed in the practice of the present invention to separate derivatized saccharides. Applicants have observed a gradual deterioration in column performance over the course of several weeks of sample analysis.

A plot of the Lucifer Yellow CH retention time over a period of nineteen days and 125 analysis are included in this report. The loss in sample retention over the test was demonstrated in a drop in sample readings taken on day 1 and day 20. For example, a reading of 16.0 in both the first and last column of day 1 (x axis = 2/8), while a reading of only about 12.8 was obtained on day 20 (x axis = 2/28). Results of Lucifer Yellow CH retention are demonstrated at Fig. 12.

The packed column was held at 50°C over the entire 20 day period studied. Overnight, the column was flushed with a buffer comprising 85% A solution (low UV PICA (0.005 M tetrabutyl ammonium phosphate, aqueous) - 15% B solution (40% CH_3 CN in low UV PICA) at 0.225 ml/min.

At weekend intervals (i.e. about every 7 days) over which the column was maintained, the column was recycled with 85% A (as above) - 15% B (as above) at 0.9 ml/min. All plunger seals were changed in the columns before final measurement of retained Lucifer Yellow Ch was made.

The same solvent program was used for each determination. This solvent program consisted of the following:

15 - 35 % B over 10 min.
35% B for 5 min.
Step to 45% B - 45% B for 5 min.
Step to 100% B - 100% B for 5 min.
5 min. linear reverse to initial conditions.

Example 7
Derivatization and Separation of a LyCH Polysaccharide Mixture

Various polysaccharides comprising mono-, di-, tri-, tetra-, penta- and hexasaccharides were derivatized with Lucifer Yellow CH. The mixture of derivatized polysaccharides were then analyzed by HPLC as described for the monosaccharides.

Results from the HPLC are presented at Table 2. As demonstrated in the Table, the individual polysaccharides of the mixture were identifiable as distinct peaks in the chromatograph (See also Fig. 14).

Table 2
Separation of LyCH Polysaccharides Mixture

hexasaccharide - peak 500 mV at 23 min.
pentasaccharide - peak 510 mV at 24 min.
tetrasaccharide - peak 520 mV at 25 min.
trisaccharide - peak 450 mV at 29 min.
disaccharide - peak 400 mV at 31 min.
monosaccharide - peak 400 mV at 34 min.

Example 8
Separation of Lucifer Yellow CH Derivatized Maltosaccharides

The present experiment was performed to demonstrate the utility of the claimed invention with malto-oligosaccharides.

The particular maltosaccharides included in this experiment were: maltohexaose, maltopentaose, maltotetraose, maltotriose, and maltose.

The Nova-pac C_{18} column was prepared as previously described. Specifically, the column was a 0.39 x 15 cm column. S.S. at 40°C. (This particular column was previously used with TPA PO_4 II R). The eluents used over the column included the following:

Eluent A - 5 mM tetrabutylammonium hydroxide titrated to pH 6.75 with acetic acid.

Eluent B - 5 mM tetrabutylammonium hydroxide in 40% (v%) acetonitrile, titrated to pH 7.4 with acetic acid.

The solvent program employed in this experiment was as follows:

Solvent Program:	Initial	1ml/min	80%A	20%B	0%C	Curve*
	12.5 min	1	55	45	0	6 (linear)
	57.5	1	40	60	0	6 (linear)
	57.6	1	0	100	0	1 (step)
	57.7	1.5	0	100	0	1 (step)

Sample 041706: 10 μl injected
Sample prepared 3/27/90
10 nanamole each maltohexaose, maltopentaose, maltotriose, maltotetraose, maltose, and glucose in 50 μl reaction buffer (0.12 M acetic acid, 0.012% tetrafluoroacetic acid) were reacted with 100 nanamole Lucifer Yellow CH (Li_2) (in 10 μl water) at 80°C for 100 min (in 3-ml Reactivial with Teflon-coated cap).

Attend of reaction time, reaction was chilled on ice, diluted to 500 μl with water, and frozen (<10°C). Sample was thawed and diluted 1 to 10 with water before HPLC. A 10 μl sample at this dilution contains 20 pmole of each derivatized species and is 0.0012 M in acetic acid and 0.00012% in TFA.

Detection: Fluorescence, ex 230 nm, > 500 nm, 0.5 at range, 0.5 spec time constant.

Results: Peak areas are comparable to those obtained for 20 pmole-derivatized glucose when analyzed using solvent program run at a flow rate of 1 ml/min.

Sample 041707: A group of monosaccharides are sh wn here for comparison purposes.
The same conditions as described for samples of maltosaccharides were mployed

to test the following samples:

Sample: 10 μl containing 20 pmole each of Lucifer Yellow CH (Li_2)-derivatized $GlcNH_2$, $GalNH_2$, Man, Gal, Glc, GalNAc, GLcNAc, Fucose.

Example 9

Electrophoretic Analysis of LyCH Monosaccharides

The present experiment was designed to demonstrate the amenability of the described saccharide derivatives to separation by the method of electrophoresis. As noted *infra*, analysis of sugars by electrophoresis is typically not possible owing to the uncharged state in which sugars exist. The presently described methods make electrophoresis a possible alternative in saccharide separation as the described process impart a charge to the molecule.

CZE (capillary zone electrophoresis) of Lucifer Yellow (CH) (Li_2) - Derivatized Monosaccharides. The buffer (final pH 8.6) used in the present analysis included the following:

20 mM $Na_2 B_4 O_7 .10 H_2O$ (aq)
20 mM Tris-Cl (pH 7)
20 mM tetrapropyl $NH_4 = H_2PO_4^-$ (pH 7)

Materials and Methods

102.7 cm Polymicro Capillary
86.7 cm to detection window
51 μm ID, 357 μm OD
30 RV (36-37 μA)
A_{210}/cm/min chart speed

An equivolume mix of ten individually derivatized monosaccharides were tested. These samples had been prepared on May 31, 1989 and stored at 5°C until run on

June 1, 1989. Approximately 0.16 pmole of each derivatized monosaccharide was loaded per sample.

The particular LyCH monosaccharides examined in this study included fucose-LyCH, glucosamine-LyCH, glucose-LyCH, LyCH-glucose, LyCH-mannose, LyCH-galactose, and N-acetyl glucosamine LyCH.

Results:

No NANA-derivative was evident in the analysis obtained. The electrophoretic analysis of the LyCH monosaccharides are presented in Fig. 15. LC separation of the LyCH monosaccharides are also presented at Fig. 16. (16A - LyCH monosaccharides at 0 time storage; 16B - LyCH monosaccharides after overnight storage, <-10°C; 16C - LyCH monosaccharides after overnight storage, 0-5°C). Applicants also include a Fast atom bombardment Mass spectrometric analysis (negative ion) using a Glycerol Matrix at Figure 17. (17A - Fucose-LyCH; 17B - Glucosamine - LyCH; 17C - Glucose-LyCH; 17D - N-acetyl glucosamine-LyCH). From these data, it appears that the derivatized monosaccharides are not readily resolved with eluent containing tetrabutyl ammonium acetate.

A maltosaccharide, for example, maltotetraose, in its maltotetraose-Lucifer Yellow CH derivatized form, is analyzed at Figure 18B. A Glc-Lucifer Yellow CH is demonstrated to have a distinct peak at about 145 (Fig. 18A - x axis). In contrast, the maltotetraose Lucifer Yellow CH demonstrates a distinct peak at about 105 (Fig. 18B - x axis).

Figure 19 demonstrates results obtained with the particular tetramer, maltotetraose in its maltotetraos LyCH form. Distinct peaks were demonstrated for the

maltotetraose-LyCH derivatives which had an additional ion-pairing reagent. For example, maltotetraose-LyCH alone produced a distinct peak at about 1100 (x axis), while maltotetraose LyCH plus one ion-pairing reagent produced a peak at about 1325 (x axis) and a maltotetraose LyCH with two ion-pairing reagents resulted in demonstration of a peak at 1575 (x axis). Figure 19 represents a mass spectra chromatograph of the tetrameres maltotetraose-LyCH, maltotetraose-LyCH with one ion pairing reagent, and maltotetraose with two ion pairing reagents. Results obtained with glucose-LyCH (a monomer) with one or two ion-pairing reagents is demonstrated at Fig. 20.

Example 10
Chromatographic Analysis of Derivatized Monosaccharides With Lucifer Yellow CH

The particular monosaccharides individually characterized in this study included: Glucose, Galactose, Mannose, Fucose, N-acetyl glucosamine, N-acetyl neuraminic acid, Glucosamine, Galactosamine, and N-acetyl galactosamine.

TABLE 3

Chromatograph Readings of Individually Derivatizied Monosaccharides

Characteristic		Concentrations		
Eluate time (appx. min.)		0.0 M/0 pnd	0.000125 M/ 50 pmole	0.00025 M/ 100 pmole
6 min.	Glucose	0	20	25
10.5 min.	Galactose	0	5(200)	5 (250)
13	Mannose	0	10	15
22.5	Fucose	60	60	60
14.5	N-Acetyl - Glucosamine	40	50	50
19.5(cum)	N-Acetyl - Neuraminic Acid	35	35	35
11 min.	Glucosamine	0	0	0
11 & 12(cum)	- Galactosamine	0	0	0
13	N-Acetyl - Galactosamine	25	25	30
Glucosamine at (1.5 Rx Tm)		Data not shown		

	0.000375 M/ 150 pmole	0.0005 M/ 200 pmole	0.00125 M/ 500 pmole	1 nmol
Glucose	30	40	50	100
Galactose	10 (500)	30 (700)	55 (1500)	100
Mannose	20	30	55	100
Fucose	60	60	60	100
N-Acetyl Glucosamine	50	60	90	100
*N-Neuraminic Acid	35	35	35	35/50
Glucosamine	0	5	10	20
*Galactosamine	0	5	25	25
N-Acetyl - Galactosamine	30	40	60	60
Glucosamine (at 1.5 Rx Tm)	Data not shown			

	0.00375 M/ 1.5 nmole	0.005 M/ 2.0 nmole	0.0125 M/ 5.0 nmole	0.025 M 10 nmole	0.05 M/ 20 nmole
Glucose	0/50	175	500	600	1100
Galactose	-	150	-	1000	1500
Mannose	150	150	500	1000	1500
Fucose	-	200	-	1100	1500
N-Acetyl - Glucosamine	150	-	500	1000	1500
N-Acetyl Neuraminic	-	40/80	100/200	100/200	-
Glucosamine	-	40	100 (90 min Rx)	500	200
Galactosamine	-	30	100	200	300
N-Acetyl - Galactosamine	-	100	400	600	1000
Glucosamine	Data not shown				

TABLE 4

Expanded Graphs - N-Acetyl Neuraminic Acid and Glucosamine Derivatives

N-Acetyl Neuraminic Acid (expanded)

Characteristic Eluate time (appx. min.)	Concentrations			
	0.00025 M	0.0005 M	0.00125 M	0.0025M
	2	5	15	25
22 min.Peak 1	2	5	15	25
23 min.Peak 2	2	5	18	30
23.5 min.Peak 3	5	10	18	40
25 min.Peak 4	38	38	80	85
27 min.Peak 5	-	-	-	-

Galactosamine (expanded)

Characteristic Eluate time (appx. min.)	Concentrations				
	50 pmole	100 pmole	150 pmole	200 pmole	500 pmole
11 min.Peak 1	40	42	30	30	60
12 min.Peak 2	40	40	30	30	75
	1000 pmole	2 nmole	5 nmole	10 nmole	20 nmole
Peak 1	85	8	21	55	90
Peak 2	84	5	28	85	80

The readings recorded in Table 3 and Table 4 were taken under A_{210}. N-acetyl neuraminic acid with Lucifer Yellow appeared as a triple peak (at between 22 and 23 minutes) and as a double peak (at 25 and 27 minutes). These particular derivatives were run a second time expanded graph under the following experimental conditions: Ex 430 nm, Em 500 nm cut-off, Range 1 μA, y-axis 0-2000 mV, Concentrations of sugar used in the reaction: 0.0025 M, 0.0005 M, 0.00125 M, 0.0025 M. Results from the expanded graph analysis of N-acetyl Neuraminic Acid and galactosamine are presented in Table 4.

Galactosamine with Lucifer Yellow CH eluted as a double peak (at about 11 and 12 minutes). This sugar was also run in an expanded additional chromatogram. Readings indicated in Table 3 and Table 4 are demonstrated graphically in chromatographs included herein in Appendix 2 and in Fig. 22.

Prophetic Example 11
Proposed Purification of Lucifer Yellow CH

The present experimental example shows a method by which the Lucifer Yellow CH is to be purified prior to its use for the derivatization of the described saccharides of the present invention. The use of purified Lucifer Yellow CH is preferred in the practice of a particularly preferred embodiment of the claimed invention, especially in order to achieve the full advantage of this derivatizing agent for chromatographic analysis of low levels of carbohydrates. Even more preferably, the derivatized sugar is also to be purified to enhance the advantageous use of the derivatizing agent.

In the proposed purification of Lucifer Yellow CH, fluorescent contaminants typically present in commercially available Lucifer Yellow CH will be removed. Lucifer Yellow CH employed in the studies described herein was of an approximately 80% purity. Lucifer Yellow CH may be employed using any of a variety of techniques, including liquid chromatography, thin layer chromatography, and electrophoresis. A Lucifer Yellow CH purity level of greater than 99% could be achieved using these purification techniques well known to those skilled in the art.

While the invention is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of the experimental examples described herein in detail. It should be understood, however, that it is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

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The following are specifically incorporated herein by reference in pertinent part for the purposes indicated.

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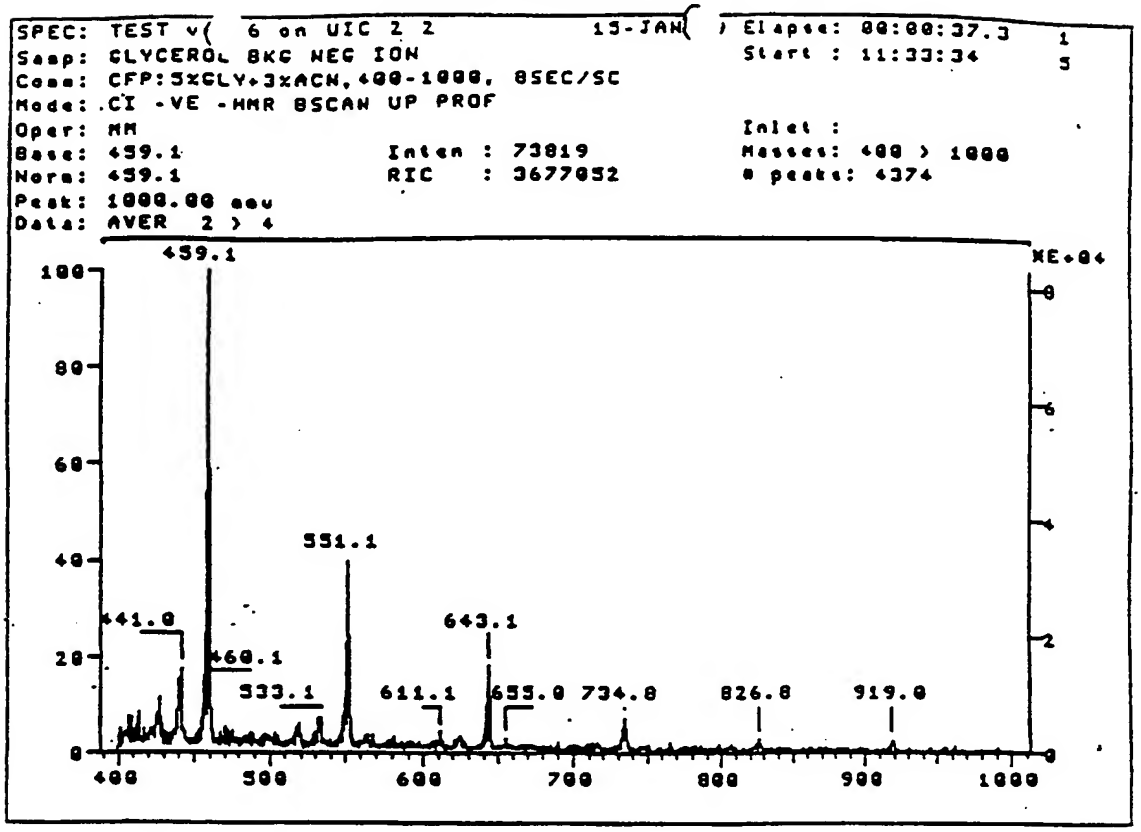
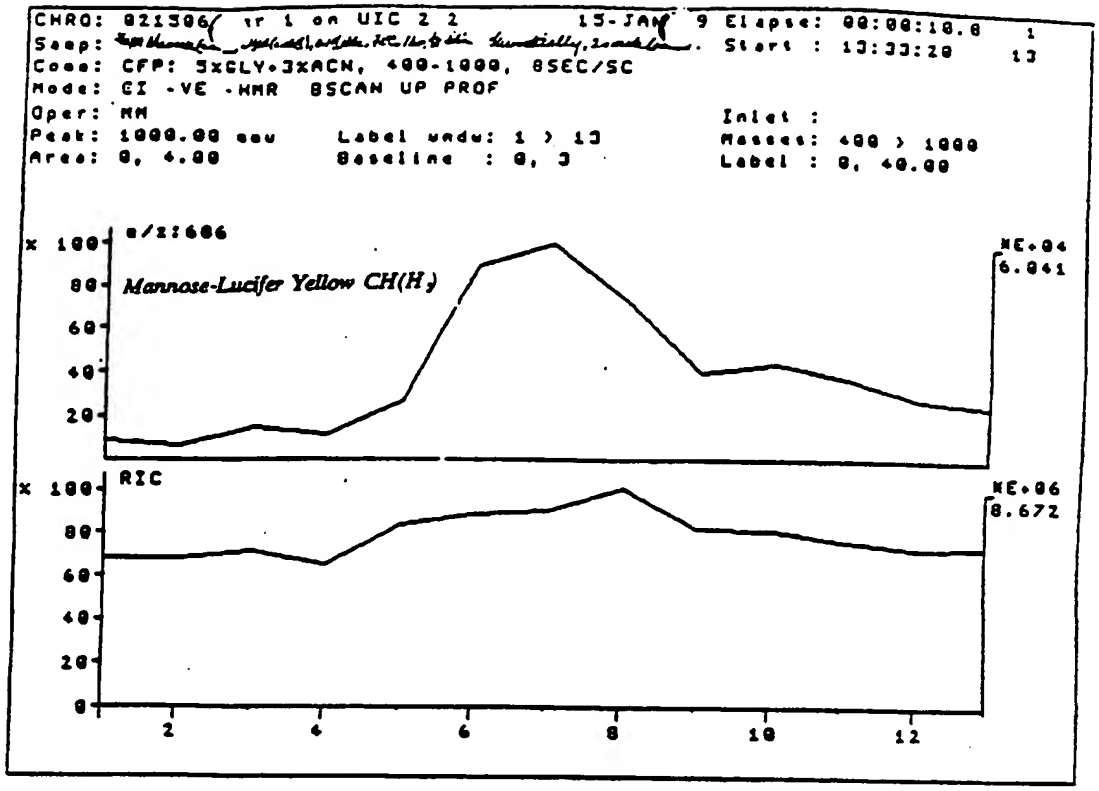
APPENDIX 1

Glycerol Background - Negative Ion

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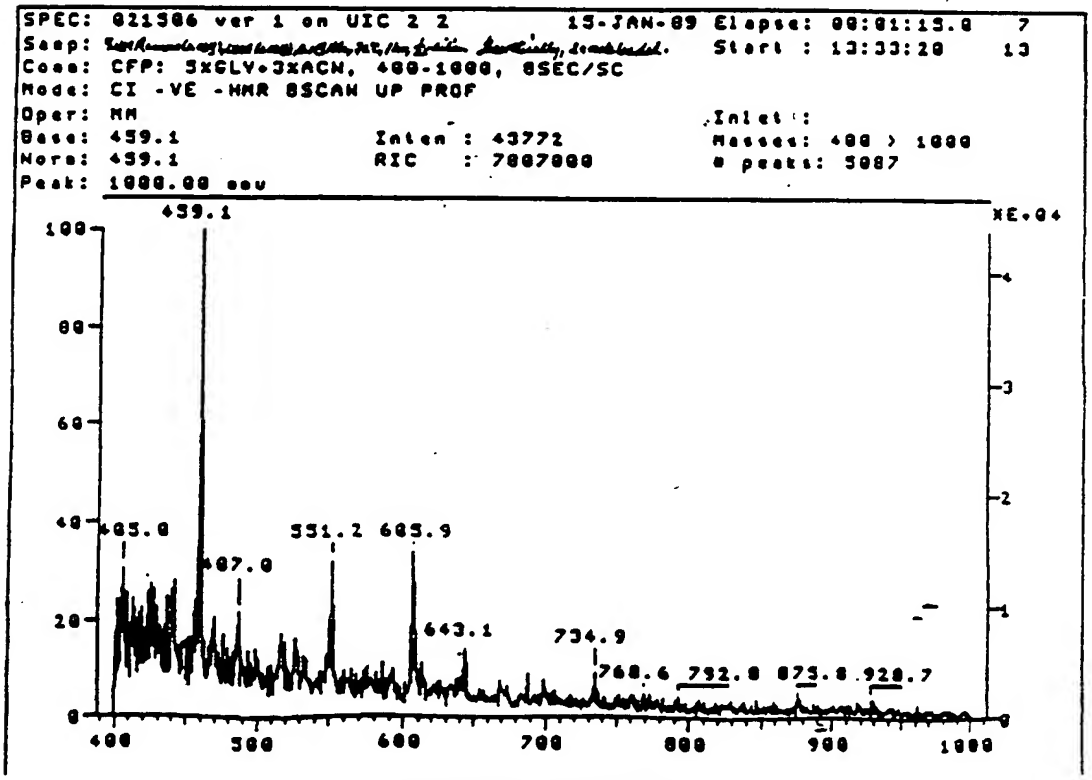
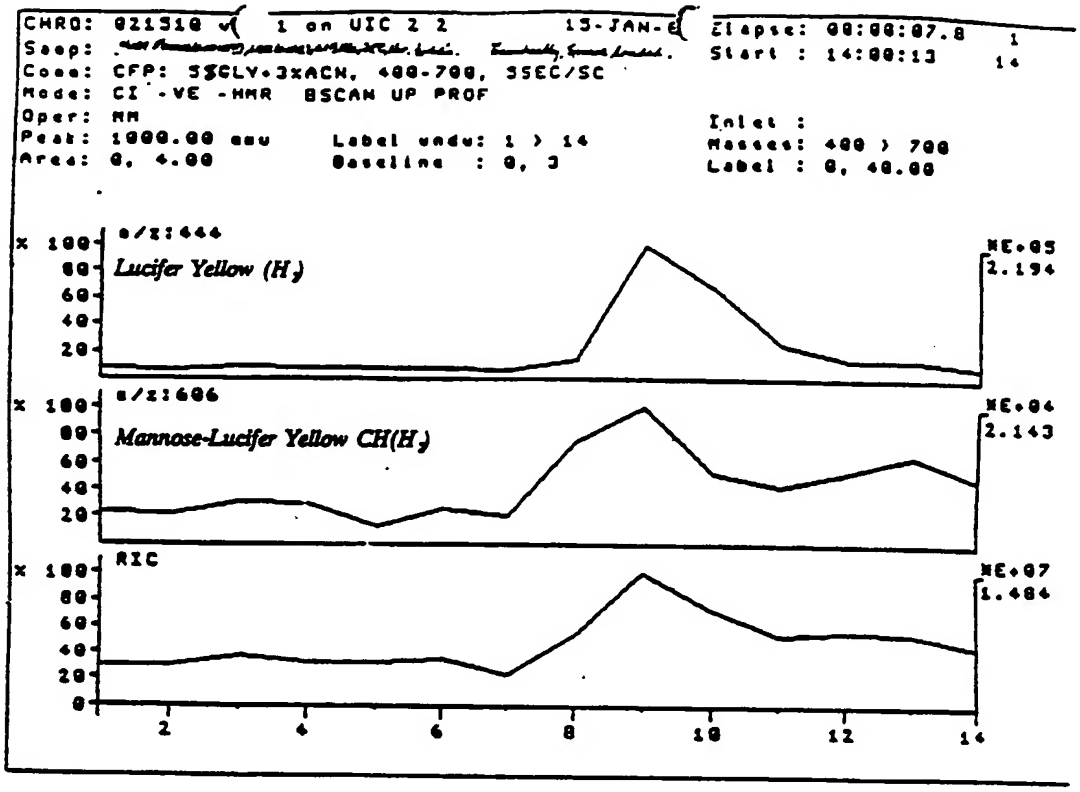
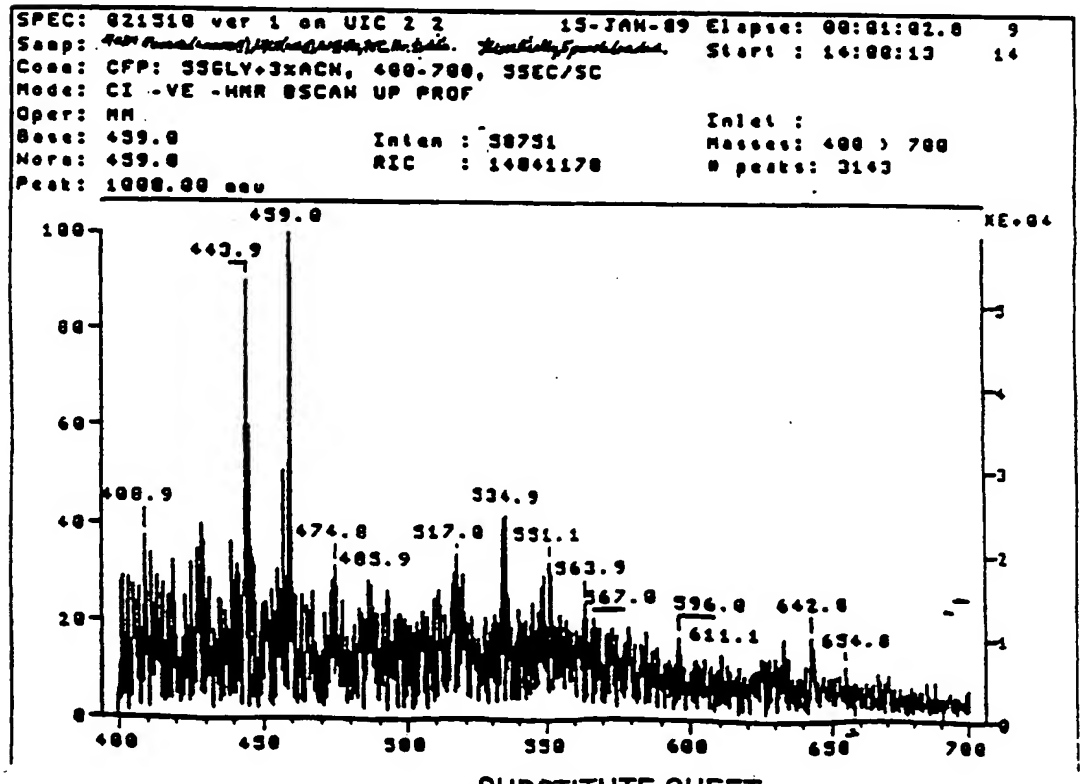
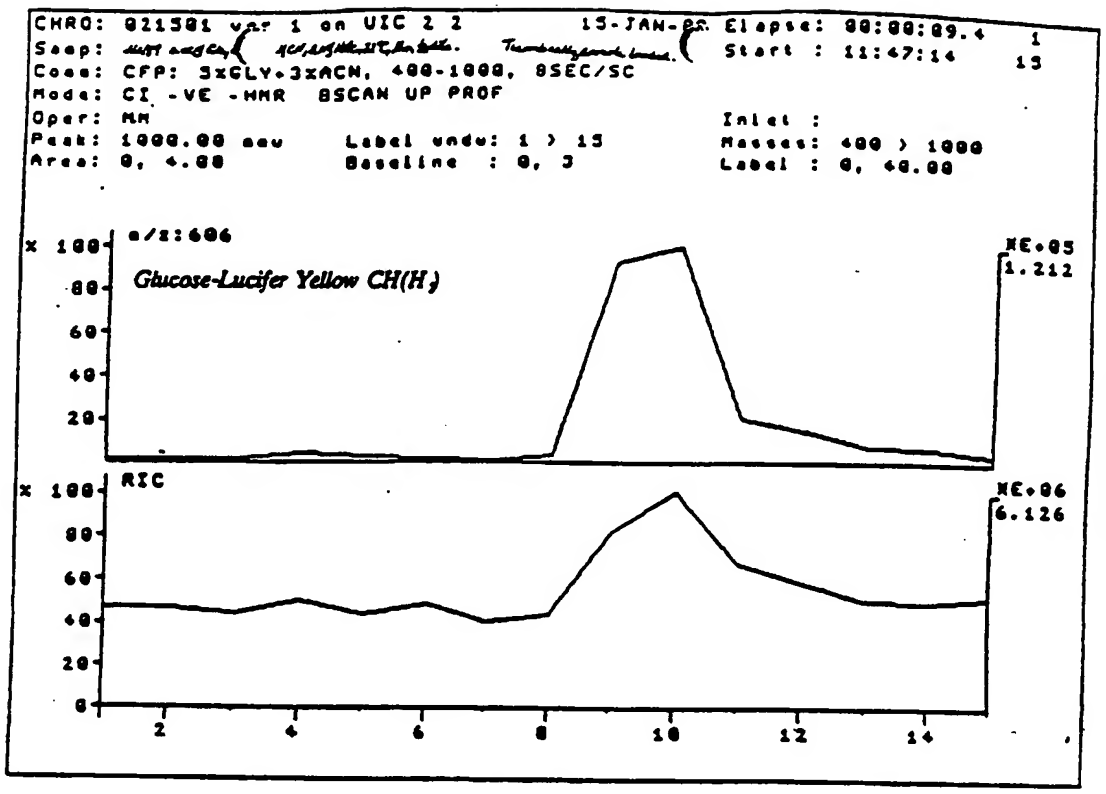
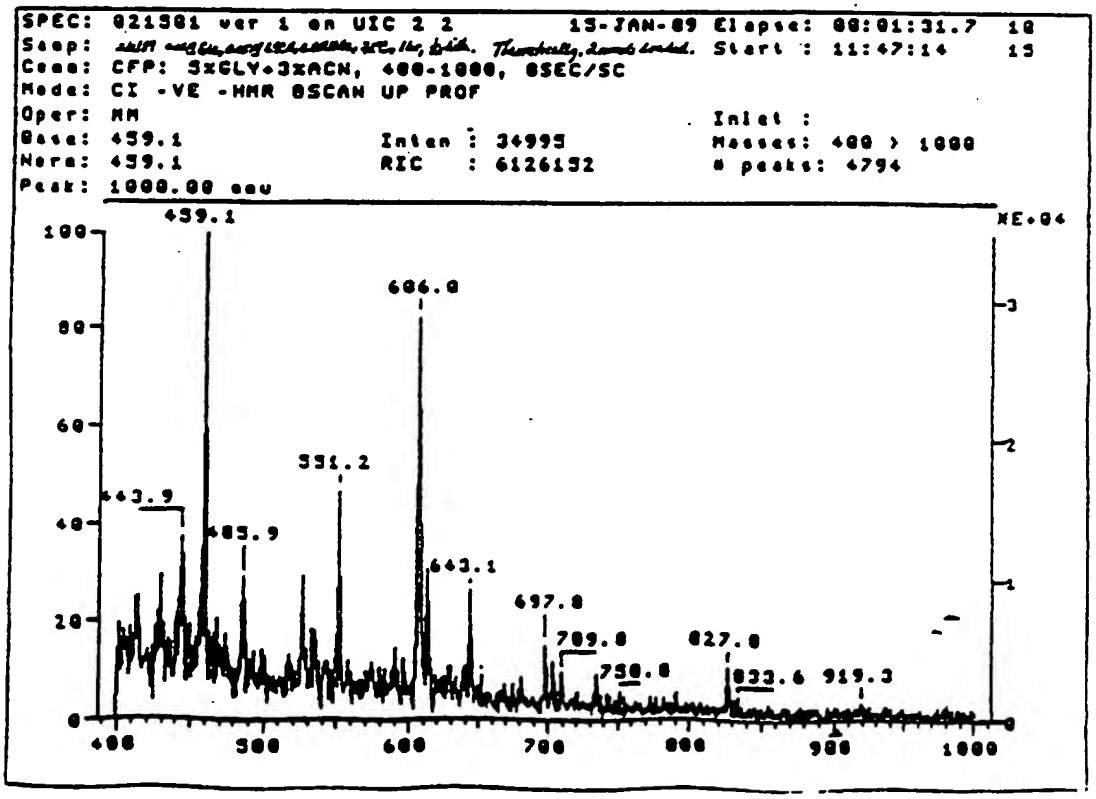
8.672

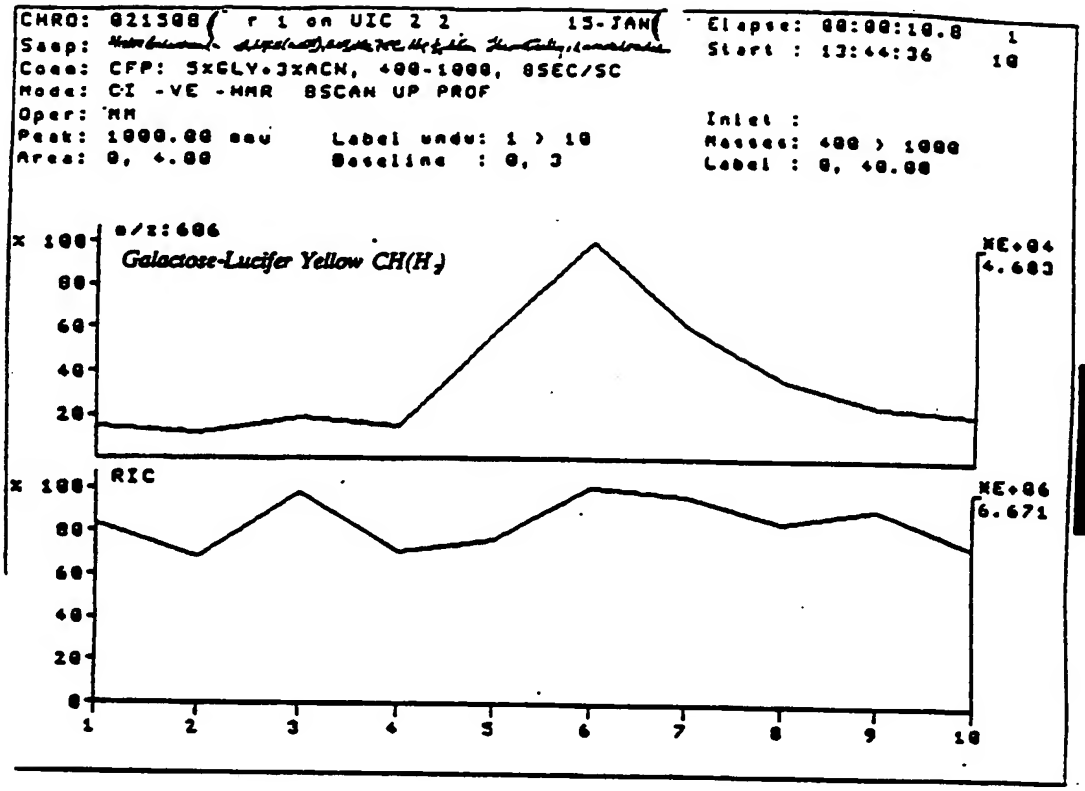

SPEC: 021306 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:01:15.0
Scan: Start : 13:33:20
Comm: CFP: 3xGLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR 8SCAN UP PROF
Oper: MM
Base: 459.1 Inten : 43772 Masses: 400 > 1000
Norm: 459.1 RIC : 7007000 # peaks: 5087
Peak: 1000.00 aau
459.1
NE+04
405.0
407.0
531.2
605.9
643.1
734.9
768.6
792.0
875.0
928.7

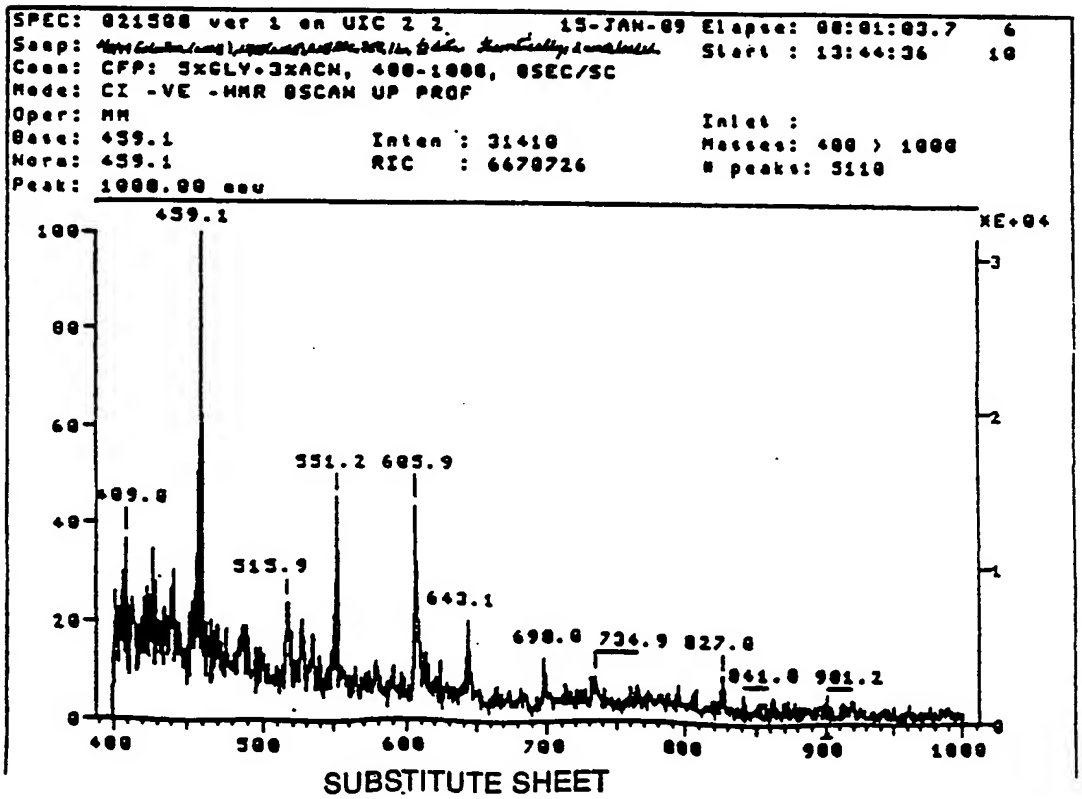

CHRO: 021318 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:00:07.8
Scan: Start : 14:00:13
Comm: CFP: 3%GLY+3xACN, 400-700, 3SEC/SC
Mode: CI -VE -HMR BSCAN UP PROF
Oper: MM
Peak: 1000.00 amu Label mode: 1 > 14
Area: 0, 4.00 Baseline : 0, 3
Inlet :
Masses: 400 > 700
Label : 0, 40.00
m/z:444
Lucifer Yellow (H2)
m/z:606
Mannose-Lucifer Yellow CH(H2)
RIC
2.194
2.143
1.484

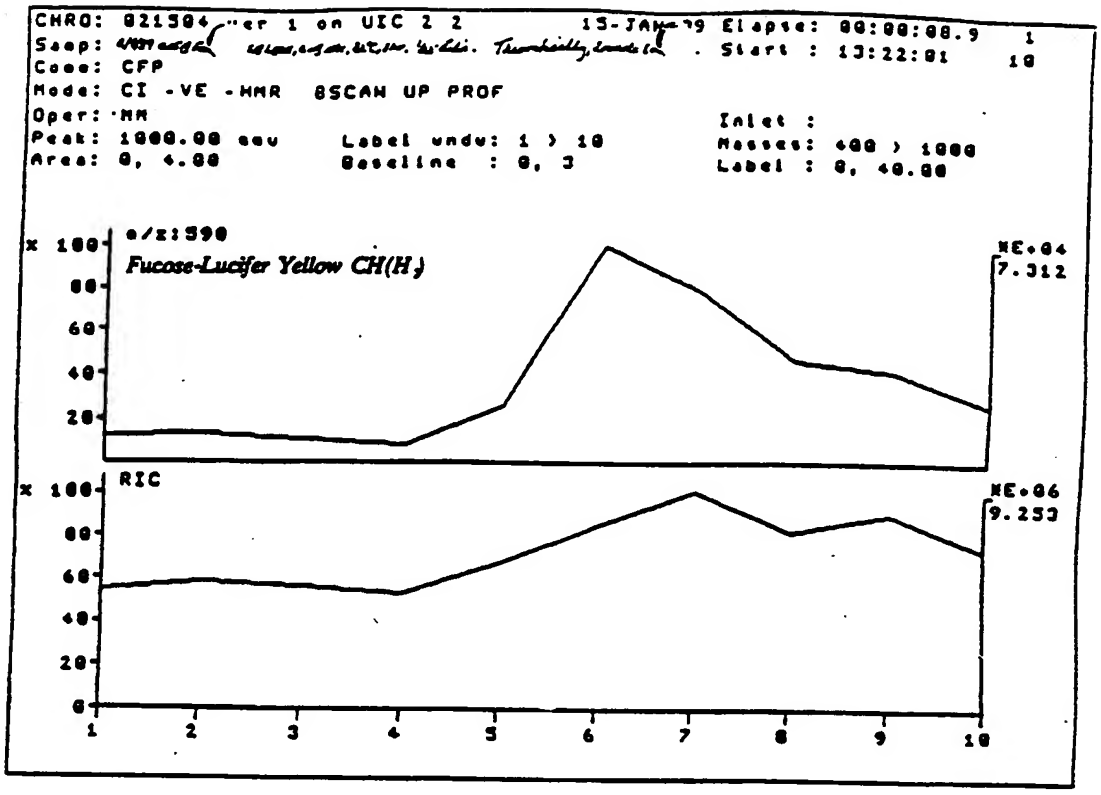

SPEC: 021318 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:01:02.8
Scan: Start : 14:00:13
Comm: CFP: 3%GLY+3xACN, 400-700, 3SEC/SC
Mode: CI -VE -HMR BSCAN UP PROF
Oper: MM
Base: 439.0 Inten : 38731
Norm: 439.0 RIC : 14841178
Peak: 1000.00 amu
Inlet :
Masses: 400 > 700
peaks: 3143
439.0
443.9
408.9
474.8
485.9
517.0
534.9
551.1
563.9
567.0
596.0
611.1
642.8
654.8

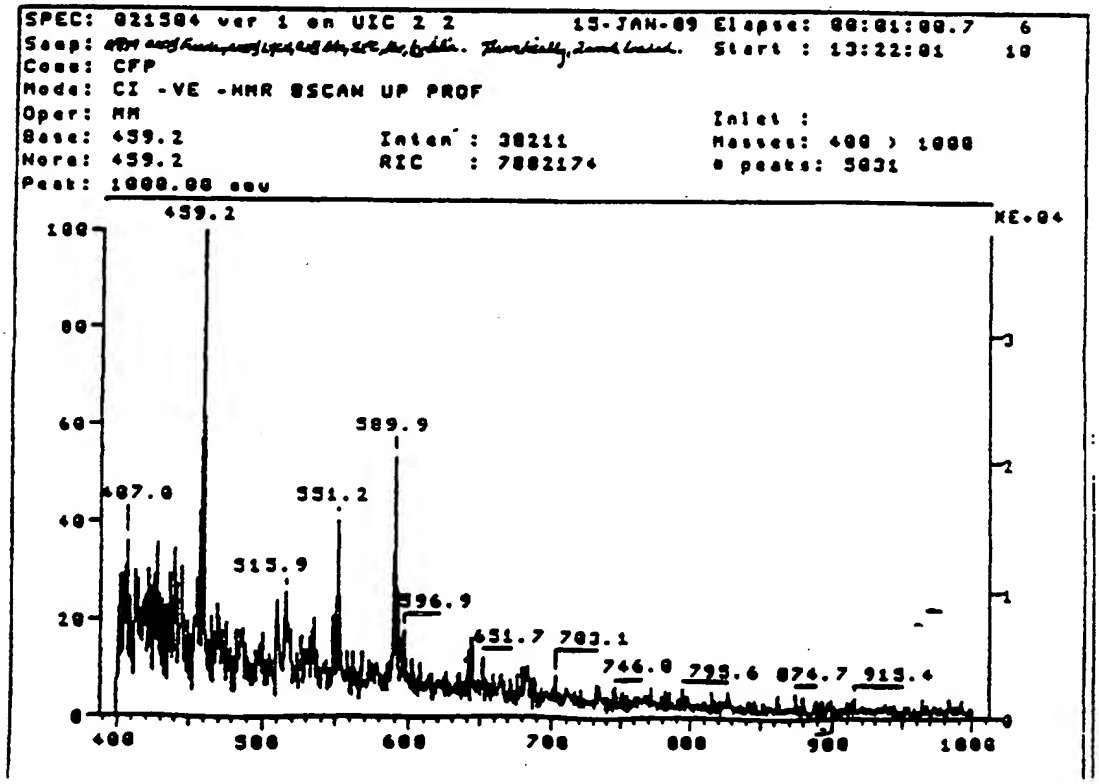

CHRO: 021501 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:00:09.4 1
Scan: Start : 11:47:14 15
Comm: CFP: 3xGLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR 8SCAN UP PROF
Oper: MM Inlet :
Peak: 1000.00 aau Label mndu: 1 > 15 Masses: 400 > 1000
Area: 0, 4.00 Baseline : 0, 3 Label : 0, 40.00
m/z:606
Glucose-Lucifer Yellow CH(H)
XE+05
1.212
RIC
XE+06
6.126

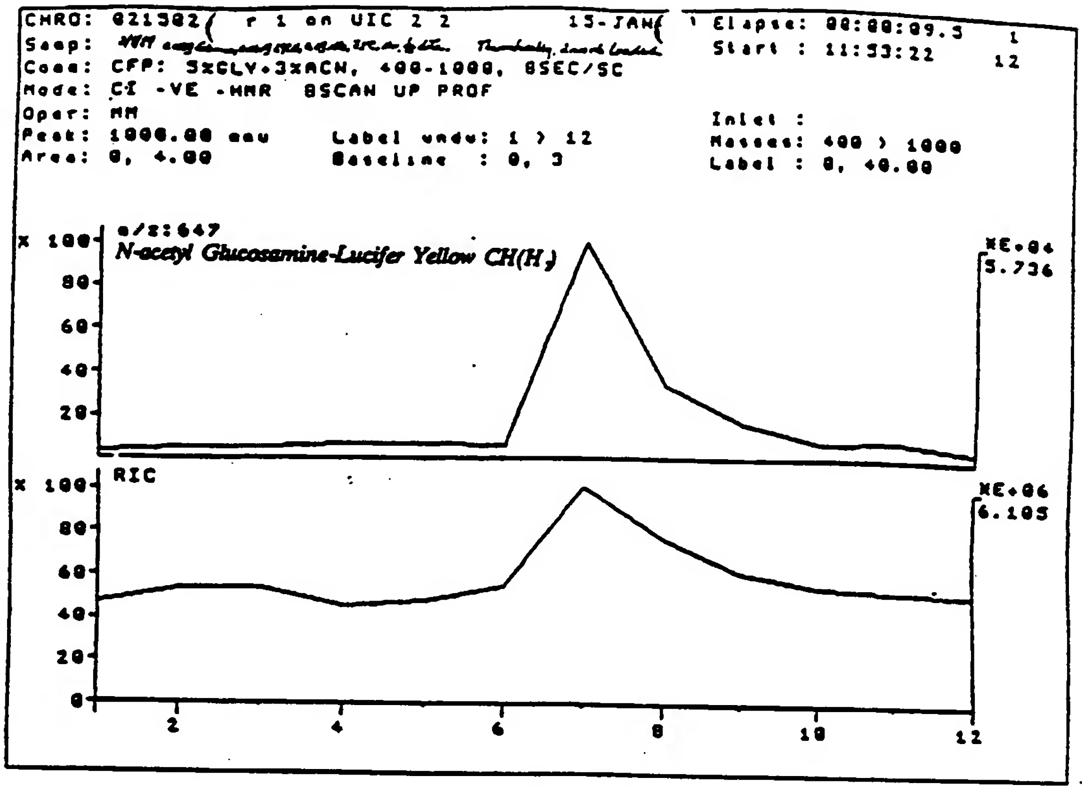

SPEC: 021501 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:01:31.7 10
Scan: Start : 11:47:14 15
Comm: CFP: 3xGLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR 8SCAN UP PROF
Oper: MM Inlet :
Base: 459.1 Inten : 34995 Masses: 400 > 1000
Norm: 459.1 RIC : 6126132 # peaks: 4794
Peak: 1000.00 aau
459.1
606.0
551.2
443.9
483.9
643.1
697.8
709.8
827.8
758.8
833.6
919.3
XE+04

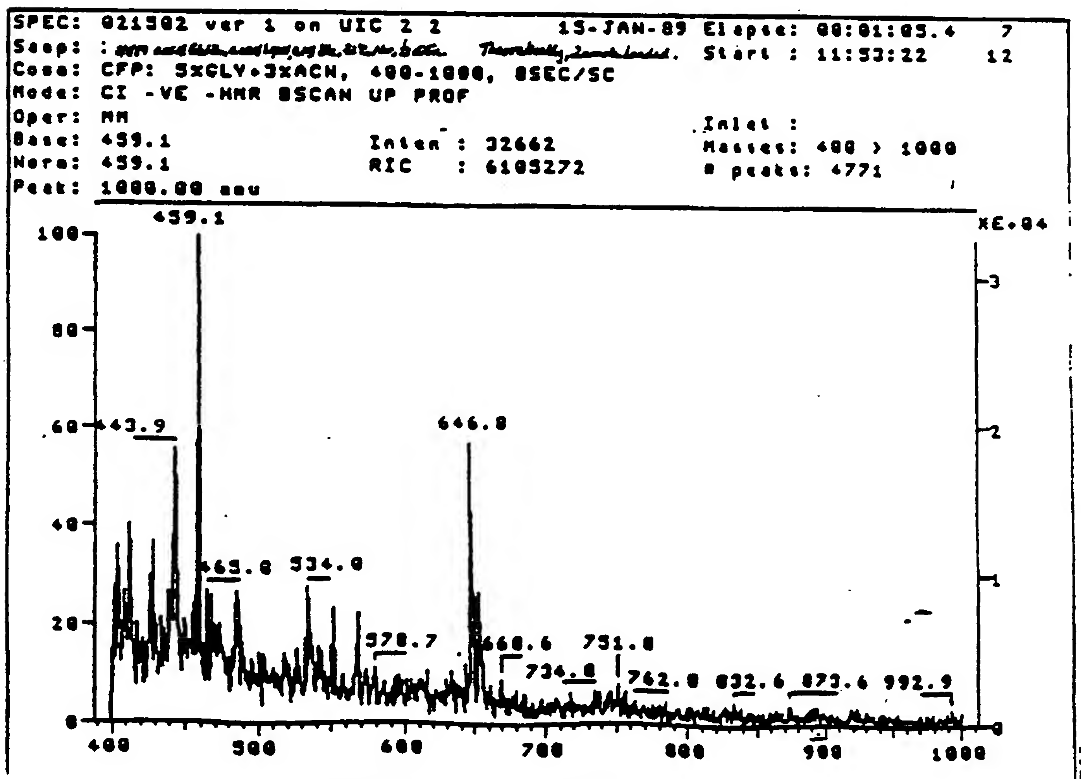

CHRO: 021508(r 1 on UIC 2 2 15-JAN(
Elapse: 00:00:10.8 1
Start : 13:44:36 10
Seap:
Ceea: CFP: 5xGLY•3xACN, 400-1000, 0SEC/SC
Mode: CI -VE -MMR 0SCAN UP PROF
Oper: MM
Peak: 1000.00 aau
Area: 0, 4.00
Label undv: 1 > 10
Baseline : 0, 3
Inlet :
Masses: 400 > 1000
Label : 0, 40.00
m/z:686
Galactose-Lucifer Yellow CH(H2)
x 100
80
60
40
20
XE+04
4.683
RIC
x 100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
XE+06
6.671

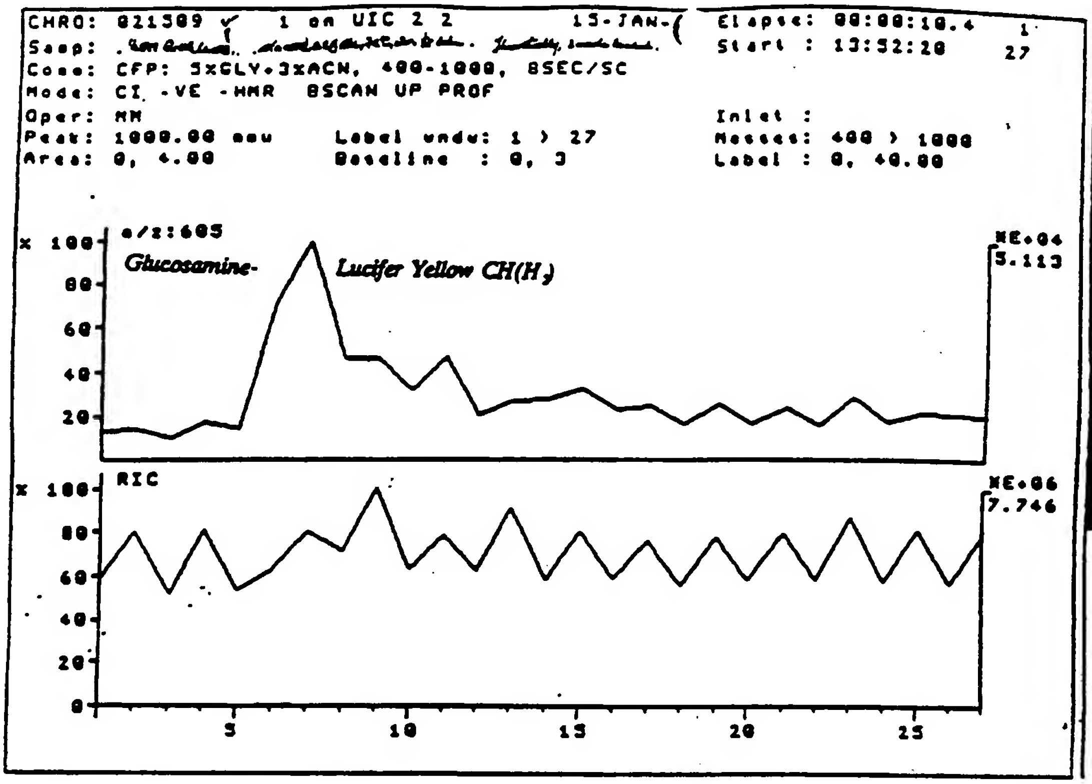

SPEC: 021508 ver 1 on UIC 2 2 15-JAN-09 Elapse: 00:01:03.7 6
Start : 13:44:36 10
Seap:
Ceea: CFP: 5xGLY•3xACN, 400-1000, 0SEC/SC
Mode: CI -VE -MMR 0SCAN UP PROF
Oper: MM
Base: 459.1
Nora: 459.1
Peak: 1000.00 aau
Inten : 31410
RIC : 6670726
Inlet :
Masses: 400 > 1000
peaks: 5110
459.1
XE+04
3
2
1
0
100
80
60
40
20
0
409.8
513.9
551.2
605.9
643.1
698.8
734.9
827.8
841.8
901.2
400
500
600
700
800
900
1000

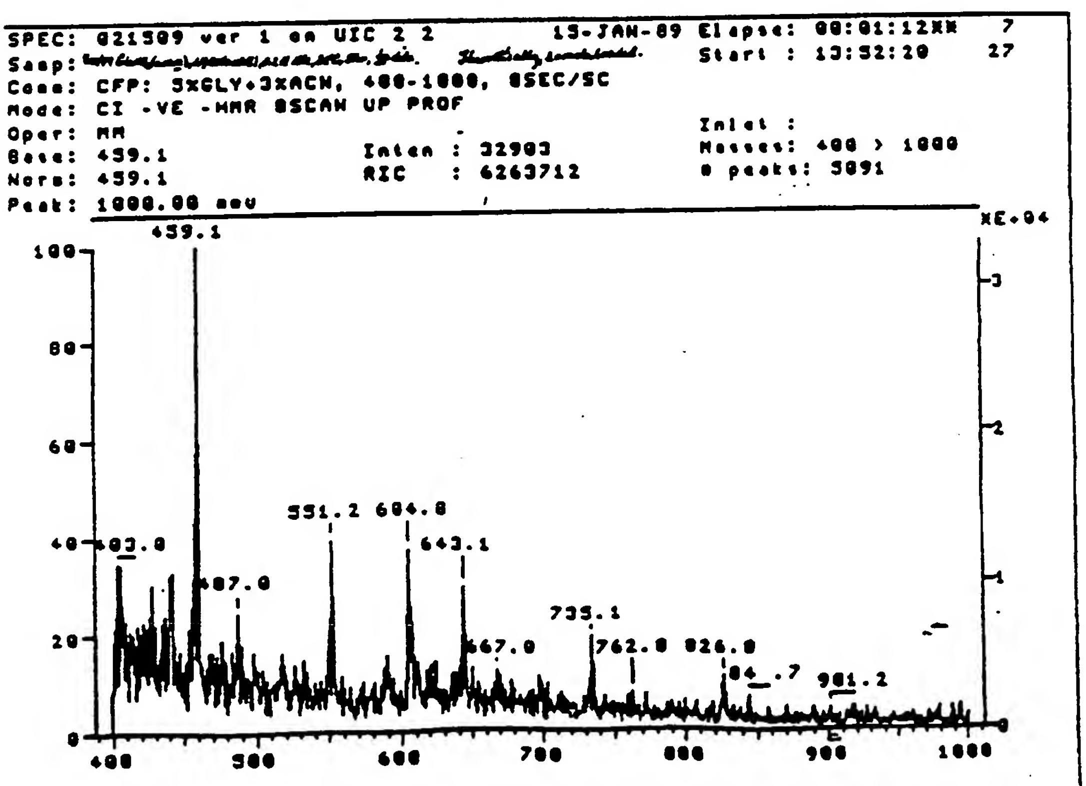

CHRO: 021504 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:00:08.9 1
Scan: Start : 13:22:01 10
Case: CFP
Mode: CI -VE -HMR BSCAN UP PROF
Oper: MM
Peak: 1000.00 aau Label undv: 1 > 10 Inlet :
Area: 0, 4.00 Baseline : 0, 3 Masses: 400 > 1000
Label : 0, 40.00
m/z:590
Fucose-Lucifer Yellow CH(H)
x 100
80
60
40
20
XE+04
7.312
RIC
x 100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
XE+06
9.253

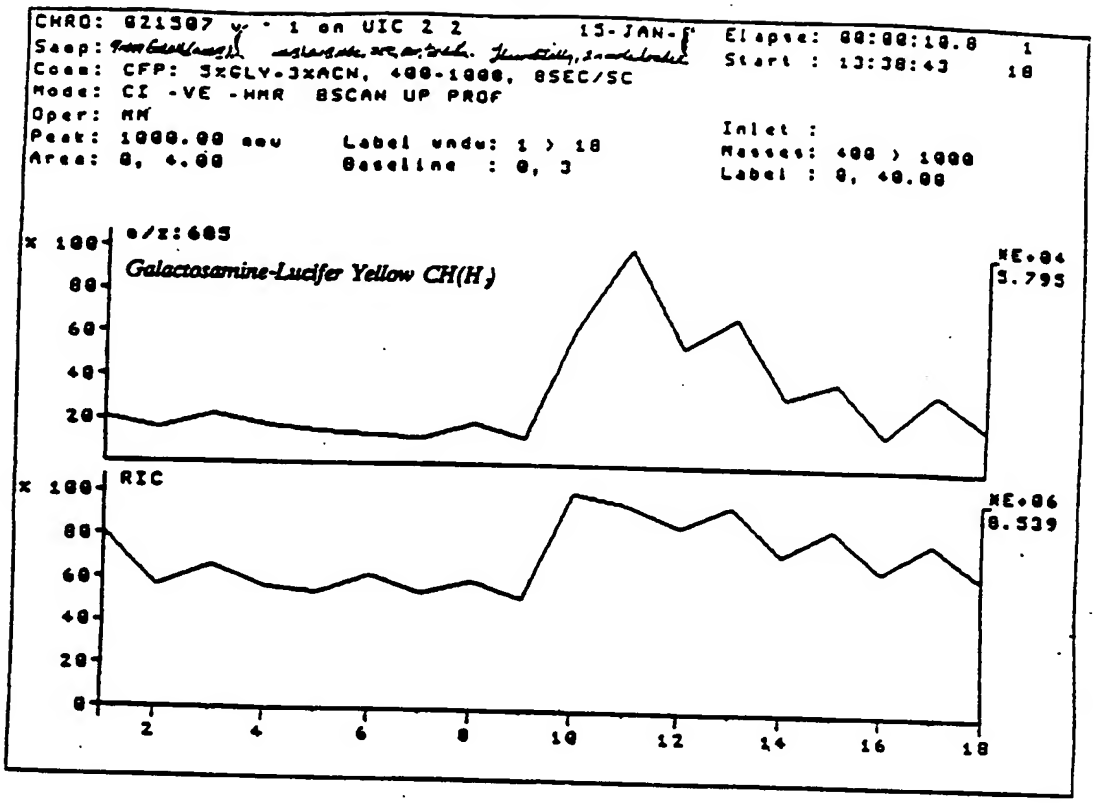

SPEC: 021504 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:01:00.7 6
Scan: Start : 13:22:01 10
Case: CFP
Mode: CI -VE -HMR BSCAN UP PROF
Oper: MM
Base: 459.2 Inten : 38211 Inlet :
Norm: 459.2 RIC : 7882174 Masses: 400 > 1000
Peak: 1000.00 aau # peaks: 5031
459.2
XE+04
100
80
60
40
20
0
589.9
487.0
551.2
515.9
596.9
631.7
703.1
746.0
795.6
874.7
915.4
400 500 600 700 800 900 1000

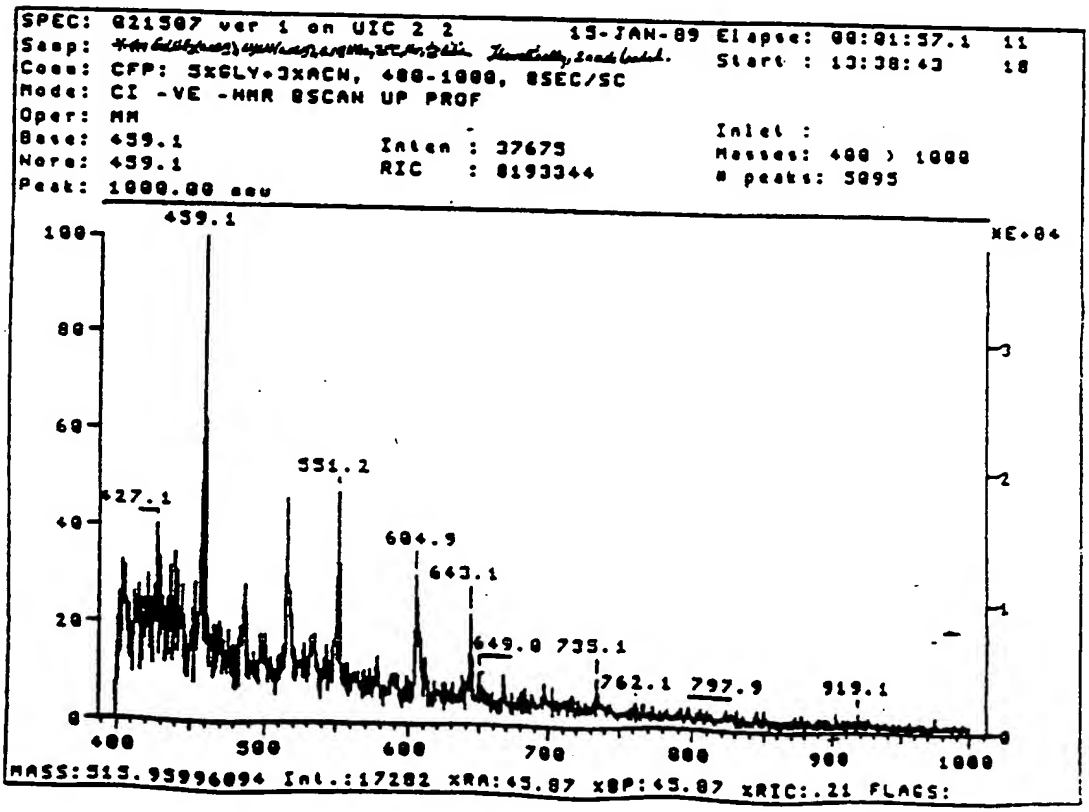

CHRO: 021382 r 1 on UIC 2 2 15-JAN
Elapse: 00:00:09.5
Start : 11:53:22
Cond: CFP: 5xCLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR BSCAN UP PROF
Oper: MM
Peak: 1000.00 amu
Area: 0, 4.00
Label wndw: 1 > 12
Baseline : 0, 3
Inlet :
Masses: 400 > 1000
Label : 0, 40.00
m/z:647
N-acetyl Glucosamine-Lucifer Yellow CH(H)
XE+04
5.736
RIC
XE+06
6.105

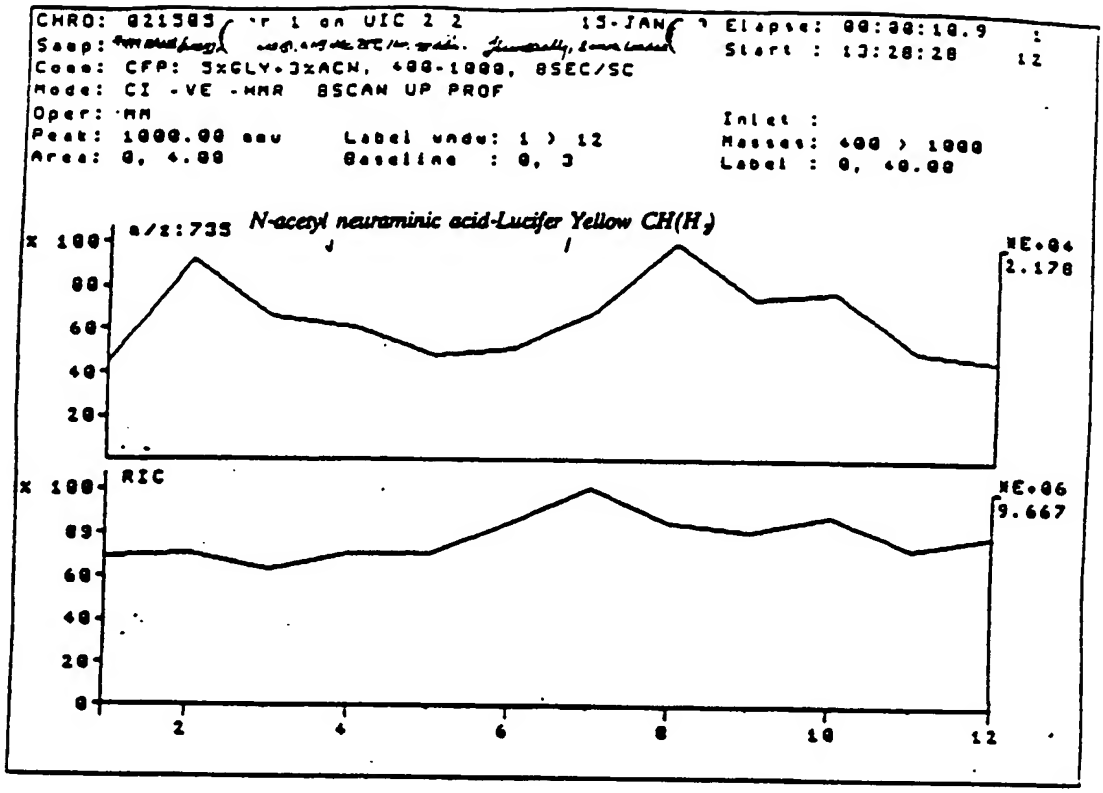

SPEC: 021382 ver 1 on UIC 2 2 15-JAN-89
Elapse: 00:01:05.4
Start : 11:53:22
Cond: CFP: 5xCLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR BSCAN UP PROF
Oper: MM
Base: 459.1
Norm: 459.1
Peak: 1000.00 amu
Inten : 32662
RIC : 6105272
Inlet :
Masses: 400 > 1000
peaks: 4771
459.1
XE+04
443.9
646.8
465.0
534.0
578.7
668.6
751.0
734.8
762.0
832.6
873.6
992.9

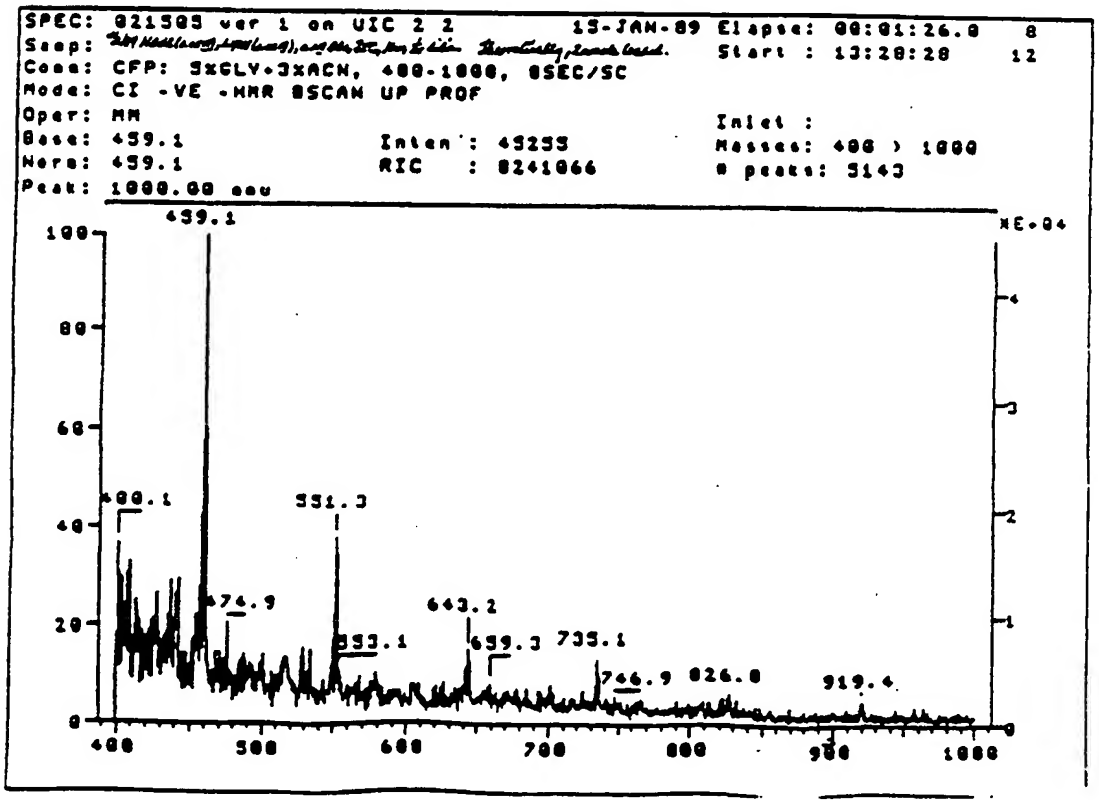

CHRO: 021589 1 on UIC 2 2 15-JAN-
Elapse: 00:00:10.4
Start : 13:52:20
1
27
Samp:
Cond: CFP: 5xGLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR BSCAN UP PROF
Oper: MM
Inlet :
Peak: 1000.00 amu
Label undo: 1 > 27
Masses: 400 > 1000
Area: 0, 4.00
Baseline : 0, 3
Label : 0, 40.00
m/z:605
Glucosamine-
Lucifer Yellow CH(H$_2$)
X 100
80
60
40
20
XE+04
3.113
RIC
X 100
80
60
40
20
0
XE+06
7.746
5
10
15
20
25

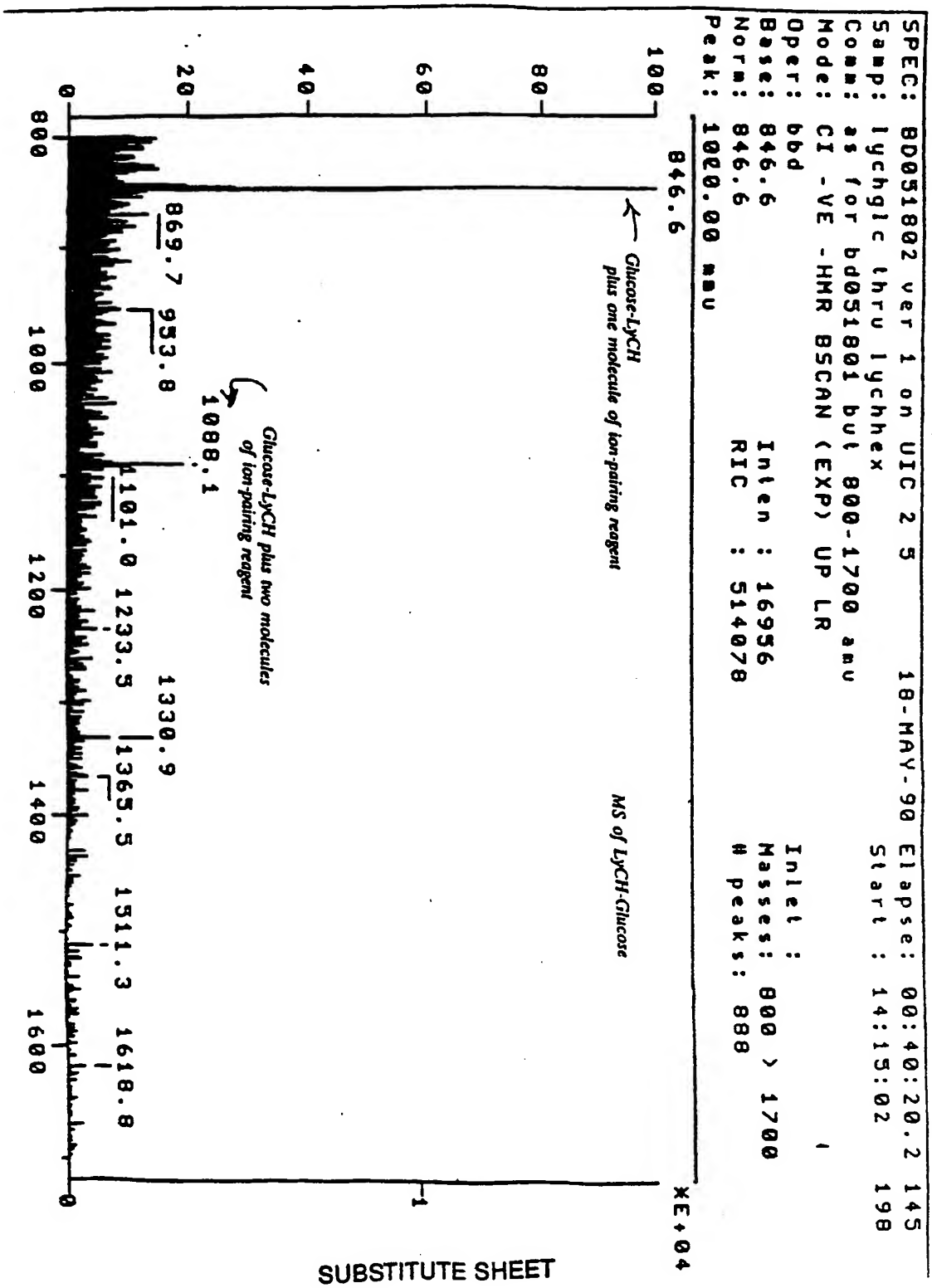

SPEC: 021589 ver 1 on UIC 2 2 15-JAN-89
Elapse: 00:01:12XX
Start : 13:52:20
7
27
Samp:
Cond: CFP: 5xGLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR BSCAN UP PROF
Oper: MM
Inlet :
Base: 459.1
Inten : 32983
Masses: 400 > 1000
Norm: 459.1
RIC : 6263712
peaks: 3891
Peak: 1000.00 amu
459.1
XE+04
100
80
60
40
20
0
3
2
1
403.0
487.0
551.2
604.8
643.1
667.0
735.1
762.8
826.8
901.2
400
500
600
700
800
900
1000


CHRO: 021507 ver 1 on UIC 2 2
15-JAN-89
Elapse: 00:00:18.8
Start : 13:38:43
Scan: 1
18
Comm: CFP: 3xGLY-3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR 8SCAN UP PROF
Oper: MM
Peak: 1000.00 aau
Area: 0, 4.00
Label wndw: 1 > 18
Baseline : 0, 3
Inlet :
Masses: 400 > 1000
Label : 0, 40.00
m/z:685
Galactosamine-Lucifer Yellow CH(H)
x 100
80
60
40
20
XE+04
3.795
RIC
x 100
80
60
40
20
0
XE+06
8.539
2
4
6
8
10
12
14
16
18

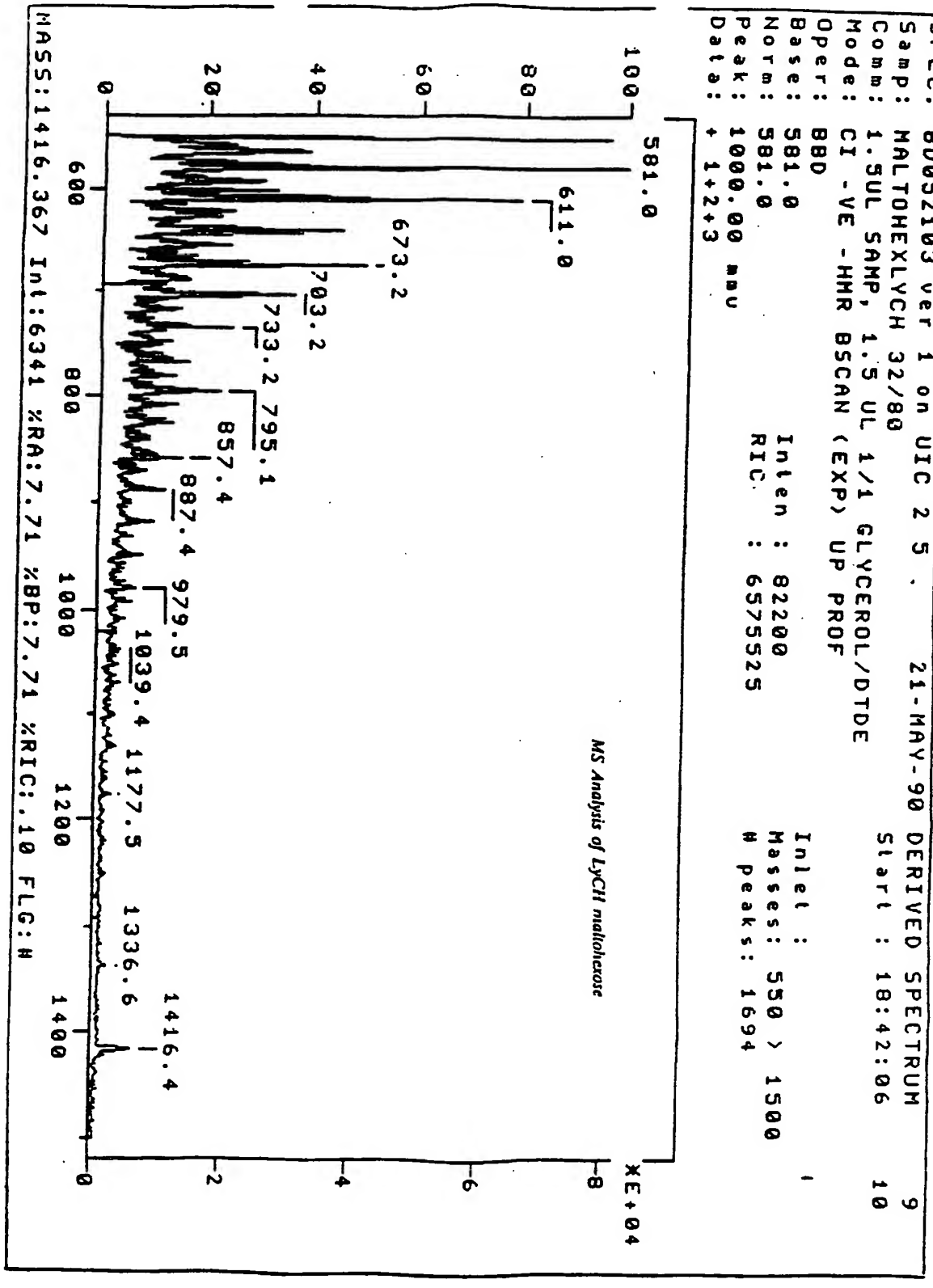

SPEC: 021507 ver 1 on UIC 2 2
15-JAN-89
Elapse: 00:01:57.1
Start : 13:38:43
Scan: 11
18
Comm: CFP: 3xGLY-3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR 8SCAN UP PROF
Oper: MM
Base: 439.1
Norm: 439.1
Peak: 1000.00 aau
Inten : 37675
RIC : 8193344
Inlet :
Masses: 400 > 1000
peaks: 5095
439.1
XE+04
100
80
60
40
20
0
427.1
551.2
604.9
643.1
649.0
735.1
762.1
797.9
919.1
400
500
600
700
800
900
1000
MASS:515.99996094 Int.:17282 %RA:45.87 %BP:45.87 %RIC:.21 FLAGS:

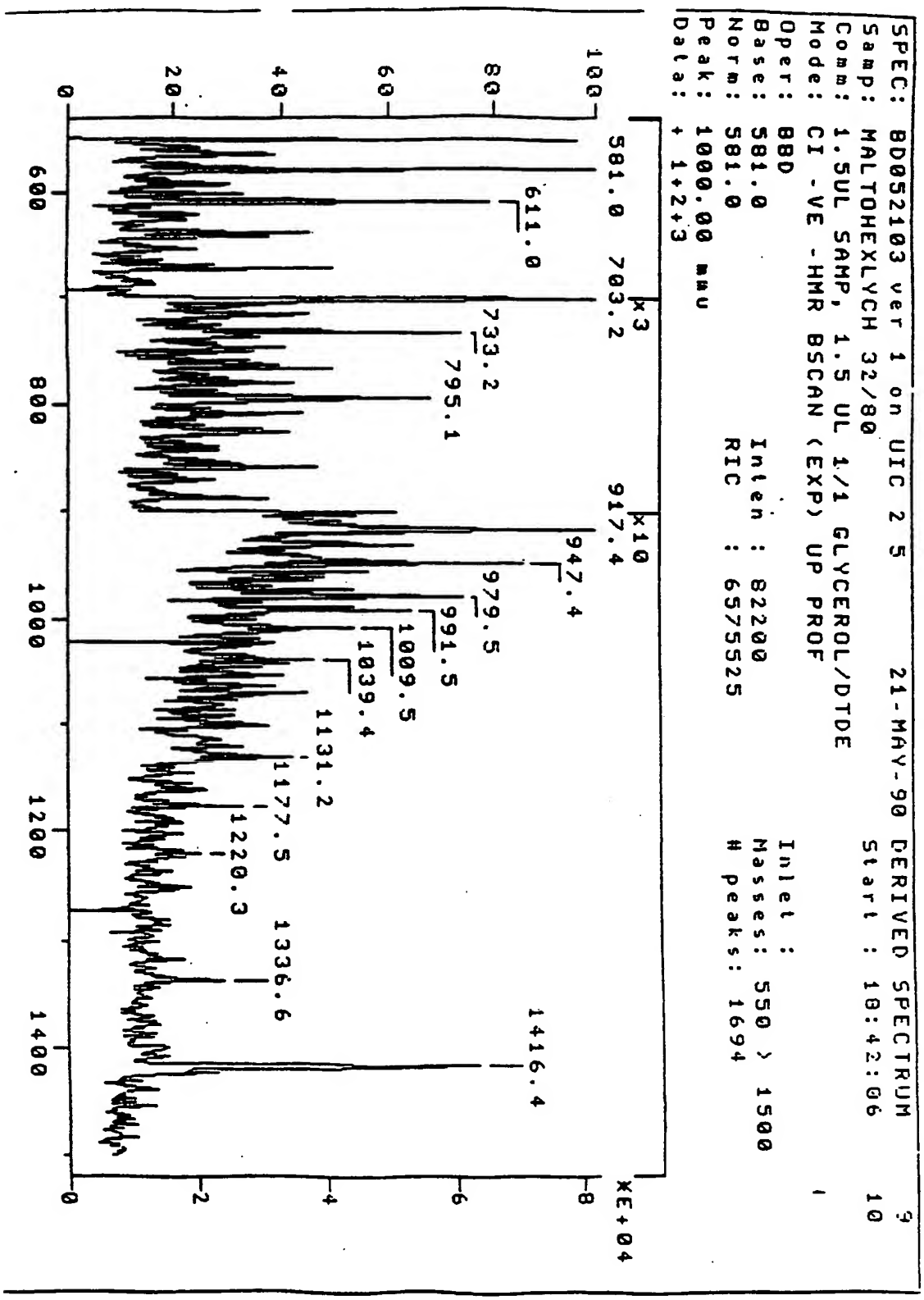

CHRO: 021589 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:00:10.9
Scan: Start : 13:28:28
Comm: CFP: 3xGLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR 8SCAN UP PROF
Oper: MM
Peak: 1000.00 aau Label unde: 1 > 12 Inlet :
Area: 0, 4.00 Baseline : 0, 3 Masses: 400 > 1000
Label : 0, 40.00
m/z:735 N-acetyl neuraminic acid-Lucifer Yellow CH(H3)
x 100
80
60
40
20
NE+04
2.178
RIC
x 100
80
60
40
20
0
NE+06
9.667
2
4
6
8
10
12

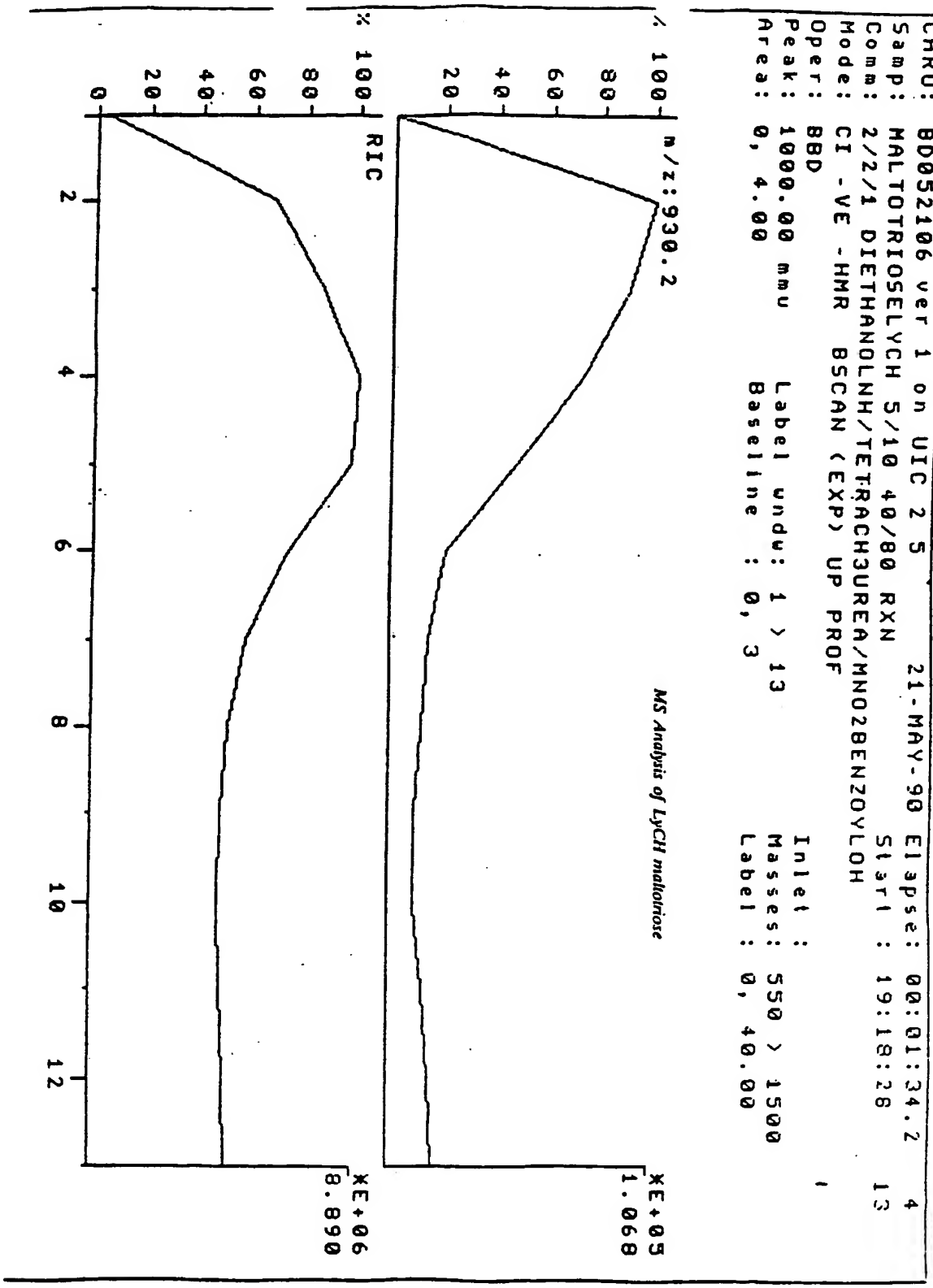

SPEC: 021589 ver 1 on UIC 2 2 15-JAN-89 Elapse: 00:01:26.0
Scan: Start : 13:28:28
Comm: CFP: 3xGLY+3xACN, 400-1000, 8SEC/SC
Mode: CI -VE -HMR 8SCAN UP PROF
Oper: MM
Base: 439.1 Inten : 43233 Inlet :
Norm: 439.1 RIC : 8241066 Masses: 400 > 1000
Peak: 1000.00 aau # peaks: 3143
439.1
NE+04
100
80
60
40
20
0
400.1
474.9
531.3
593.1
643.2
699.3
735.1
766.9
826.8
919.4
400
500
600
700
800
900
1000



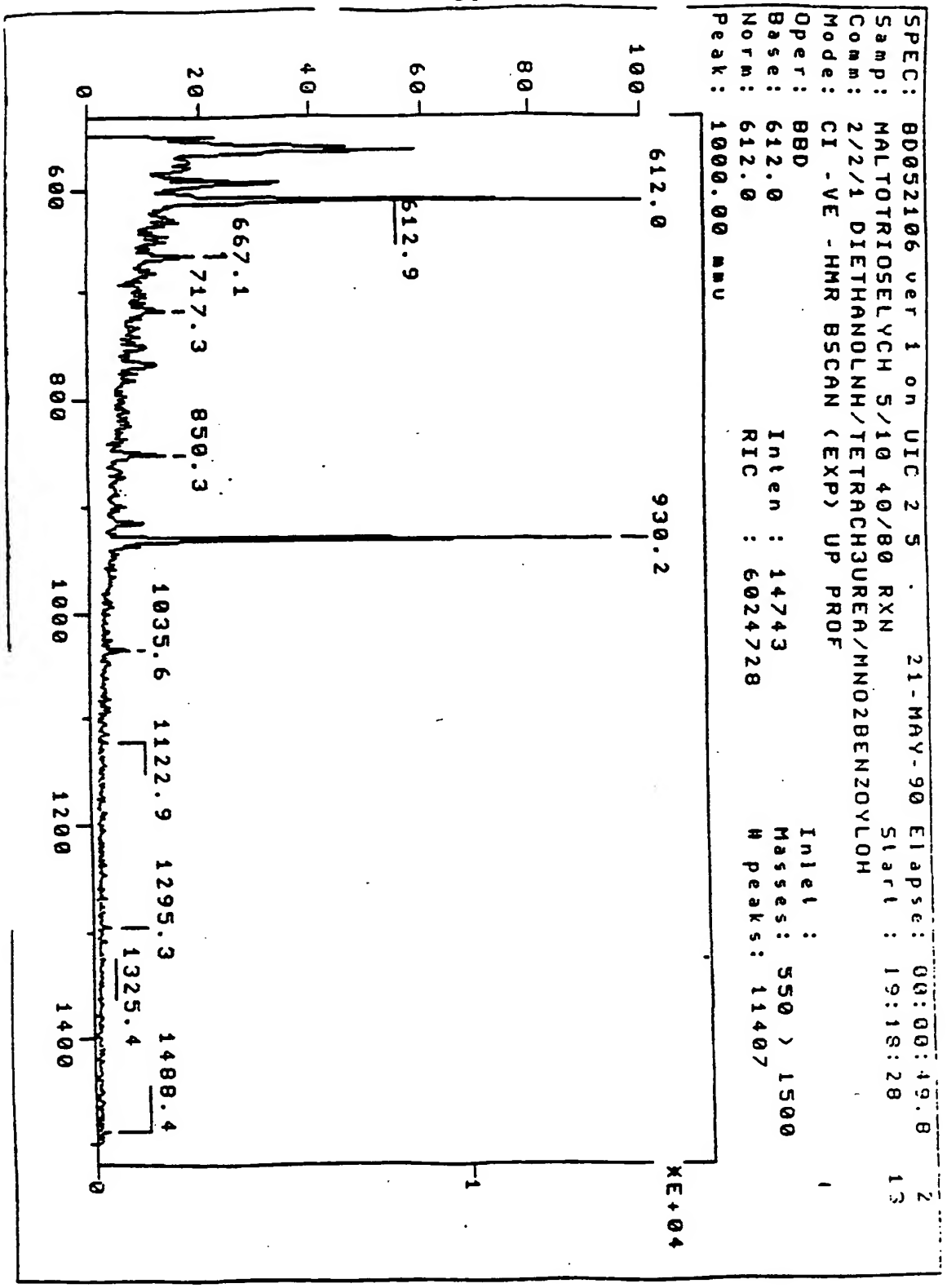
SUBSTITUTE SHEET

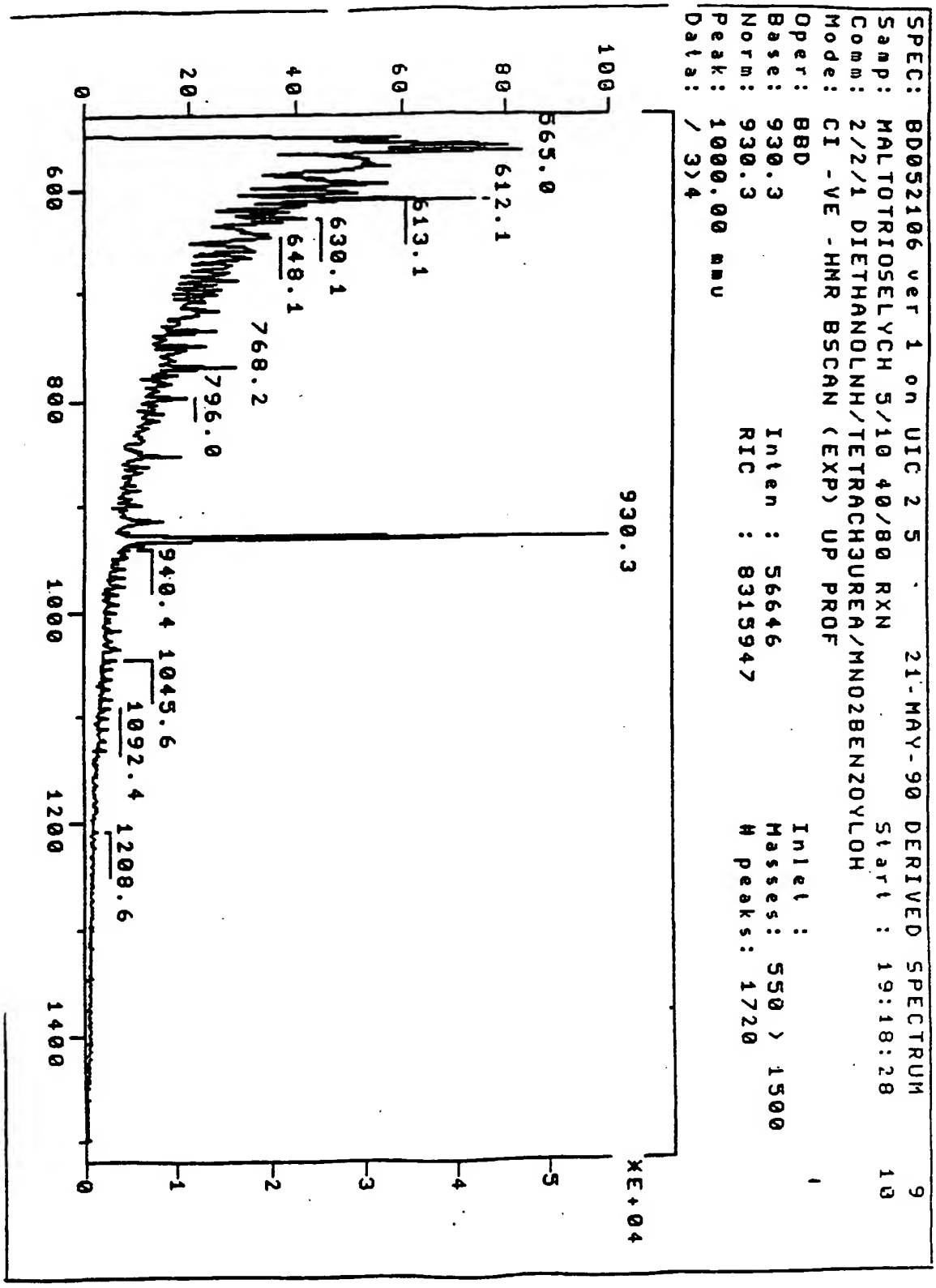

SPEC: BD052103 ver 1 on UIC 2 5 . 21-MAY-90 DERIVED SPECTRUM 9
Samp: MALTOHEXLYCH 32/80 Start : 18:42:06 10
Comm: 1.5UL SAMP, 1.5 UL 1/1 GLYCEROL/DTDE
Mode: CI -VE -HMR BSCAN (EXP) UP PROF
Oper: BBD
Base: 581.0 Inlet : 82200
Norm: 581.0 RIC : 6575525 Masses: 550 > 1500
Peak: 1000.00 mmu # peaks: 1694
Data: + 1+2+3
581.0
611.0
673.2
703.2
733.2
795.1
857.4
887.4
979.5
1039.4
1177.5
1336.6
1416.4
MS Analysis of LyCH maltohexose
MASS:1416.367 Int:6341 %RA:7.71 %BP:7.71 %RIC:.10 FLG:N
100
80
60
40
20
0
600
800
1000
1200
1400
8
6
4
2
0
XE+04

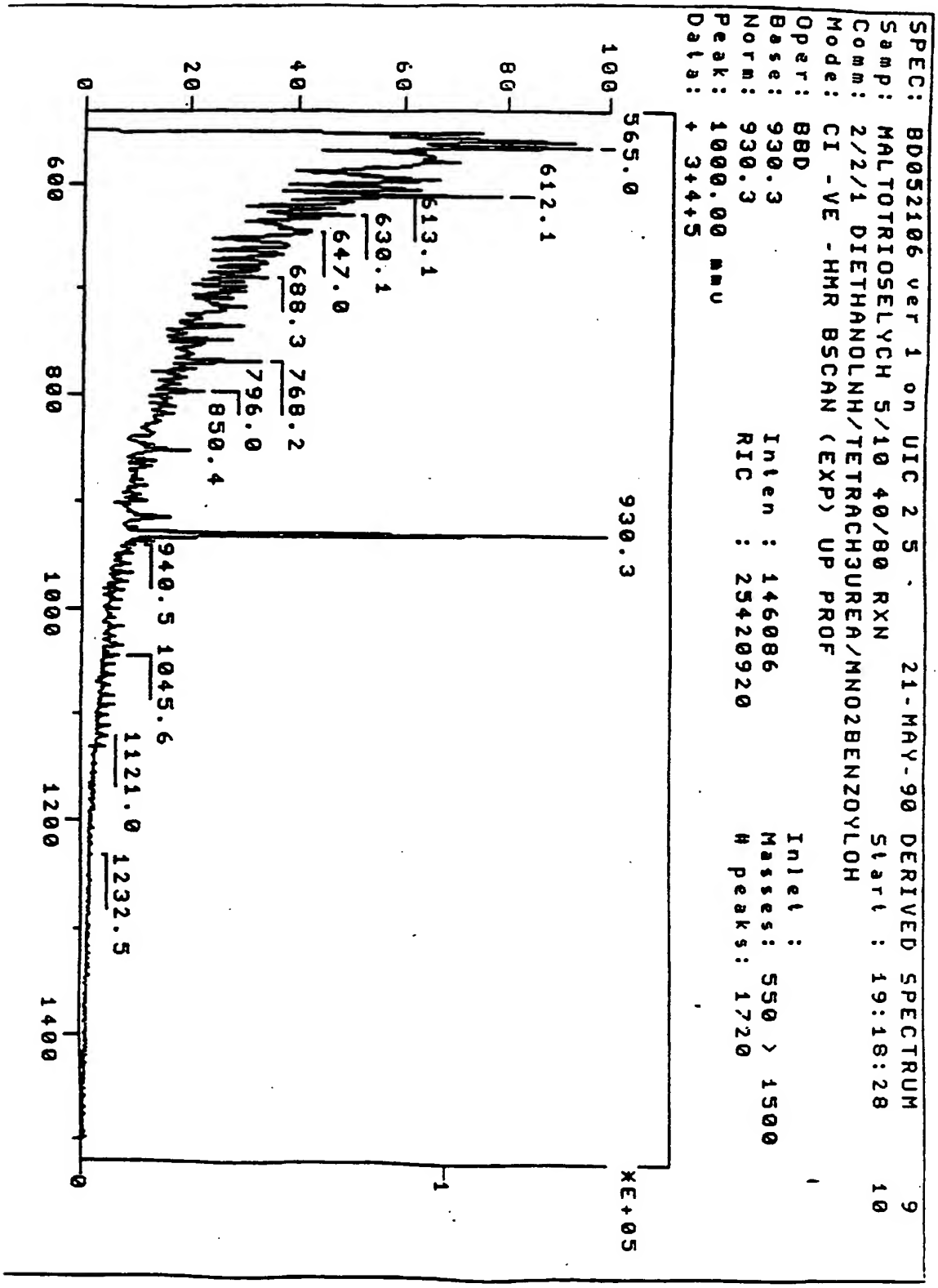

SPEC: BD052103 ver 1 on UIC 2 5 21-MAY-90 DERIVED SPECTRUM 9
Samp: MALTOHEXLYCH 32/80 Start : 10:42:06 10
Comm: 1.5UL SAMP, 1.5 UL 1/1 GLYCEROL/DTDE
Mode: CI -VE -HMR BSCAN (EXP) UP PROF 1
Oper: BBD Inlet :
Base: 581.0 Inten : 82200 Masses: 550 > 1500
Norm: 581.0 RIC : 6575525 # peaks: 1694
Peak: 1000.00 mmu
Data: + 1+2+3
581.0
703.2
x3
611.0
733.2
795.1
917.4
x10
947.4
979.5
991.5
1009.5
1039.4
1131.2
1177.5
1220.3
1336.6
1416.4
XE+04



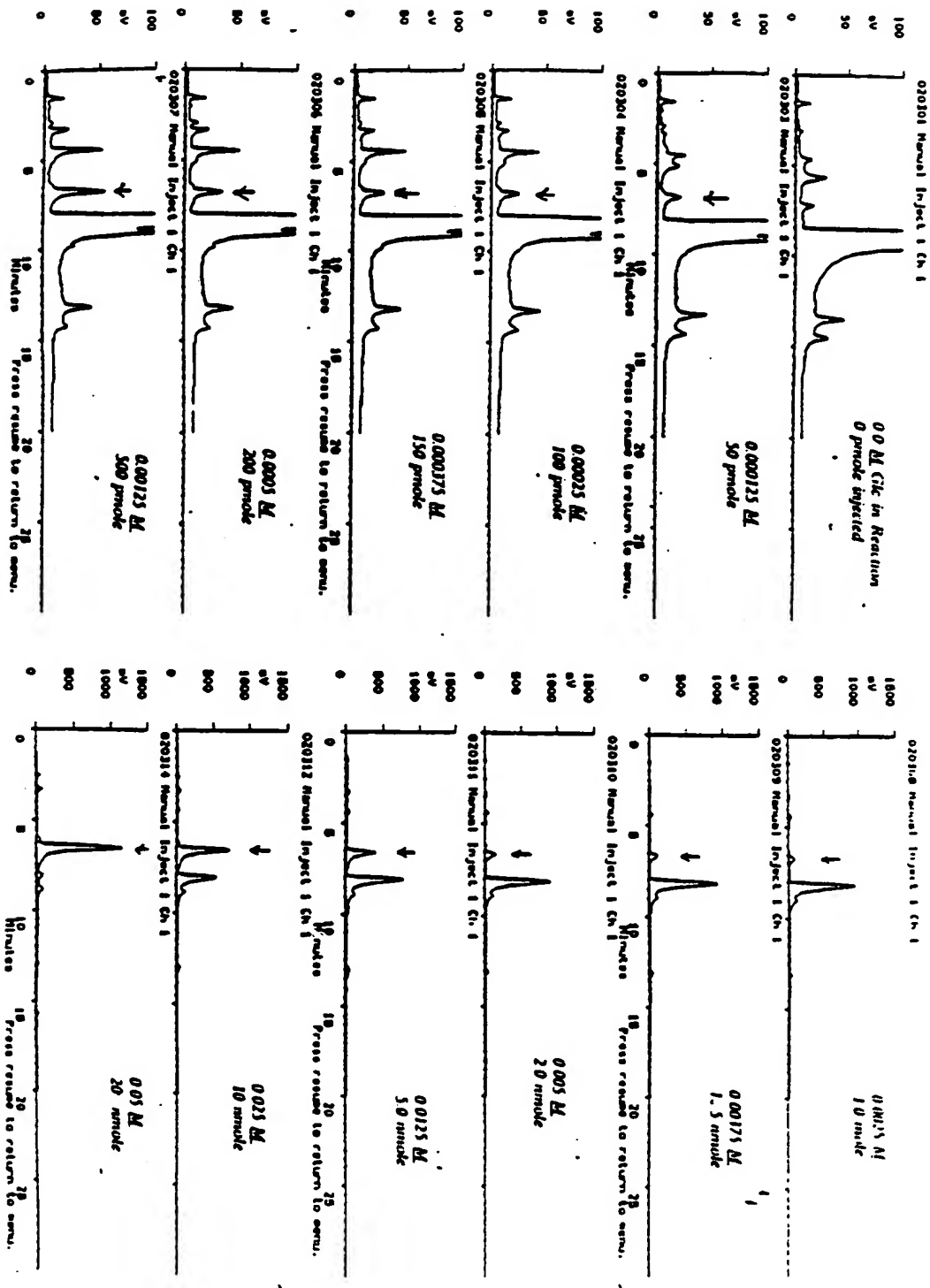
SUBSTITUTE SHEET


SPEC: BD052106 ver 1 on UIC 2 5 21-MAY-90 Elapse: 00:00:49.8
Samp: MALTOTRIOSELYCH 5/10 40/80 RXN Start : 19:18:28
Comm: 2/2/1 DIETHANOLNH/TETRACH3UREA/MNO2BENZOYLOH
Mode: CI -VE -HMR BSCAN (EXP) UP PROF
Oper: BBD Inlet :
Base: 612.0 Inten : 14743 Masses: 550 > 1500
Norm: 612.0 RIC : 6024728 # peaks: 11407
Peak: 1000.00 mmu
612.0
612.9
667.1
717.3
850.3
930.2
1035.6
1122.9
1295.3
1325.4
1488.4
XE+04

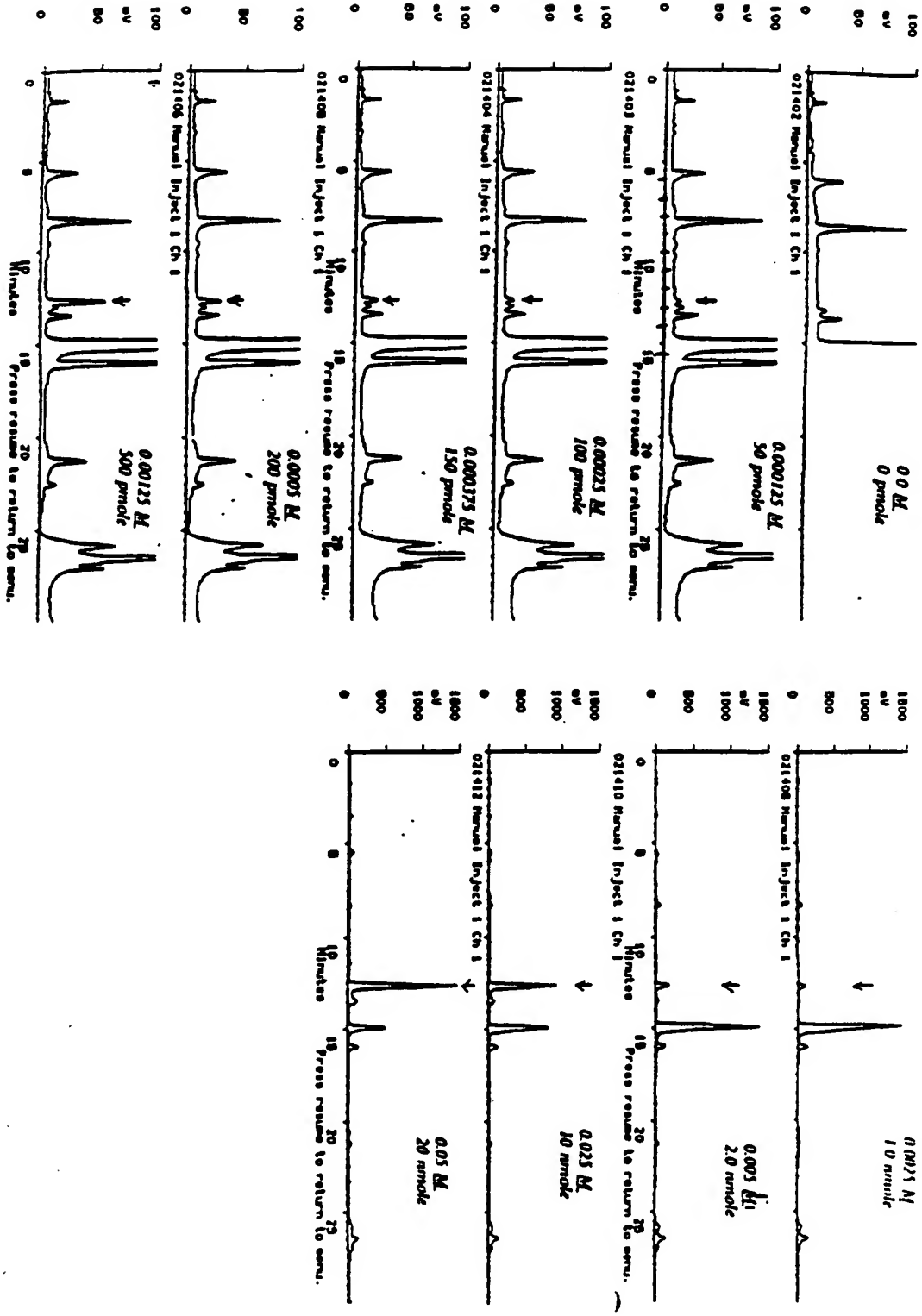

SPEC: BD052106 ver 1 on UIC 2 5 21-MAY-90 DERIVED SPECTRUM 9
Samp: MALTOTRIOSELYCH 5/10 40/80 RXN Start : 19:18:28 10
Comm: 2/2/1 DIETHANOLNH/TETRACH3UREA/MNO2BENZOYLOH
Mode: CI -VE -HNR BSCAN (EXP) UP PROF
Oper: BBD Inlet :
Base: 930.3 Inten : 56646 Masses: 550 > 1500
Norm: 930.3 RIC : 8315947 # peaks: 1720
Data: / 3>4
565.0
612.1
613.1
630.1
648.1
768.2
796.0
930.3
940.4
1045.6
1092.4
1208.6
XE+04


SPEC: BD052106 ver 1 on UIC 2 5 . 21-MAY-90 DERIVED SPECTRUM 9
Samp: MALTOTRIOSELYCH 5/10 40/80 RXN Start : 19:18:28 10
Comm: 2/2/1 DIETHANOLNH/TETRACH3UREA/MNO2BENZOYLOH
Mode: CI -VE -HMR BSCAN (EXP) UP PROF
Oper: BBD Inlet :
Base: 930.3 Inlen : 146086 Masses: 550 > 1500
Norm: 930.3 RIC : 25420920 # peaks: 1720
Peak: 1000.00 mmu
Data: + 3+4+5
565.0
612.1
613.1
630.1
647.0
688.3
768.2
796.0
850.4
930.3
940.5
1045.6
1121.0
1232.5
*E+05

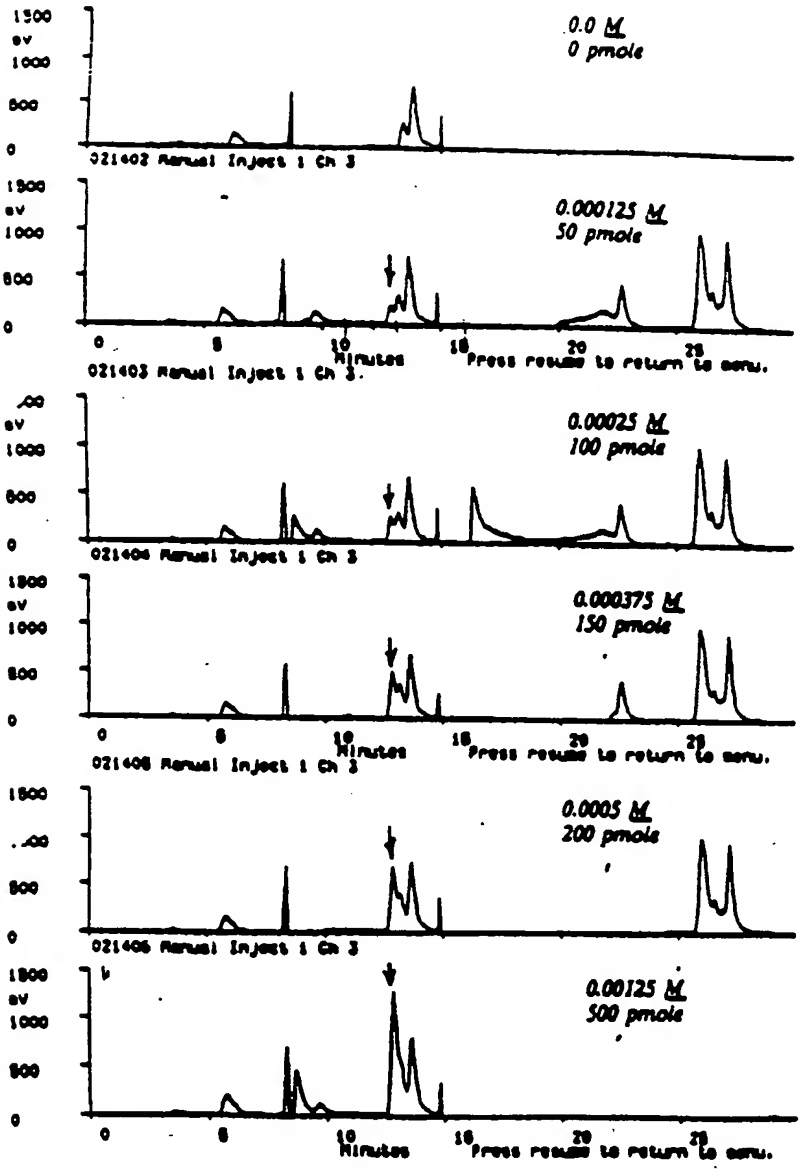
APPENDIX 2



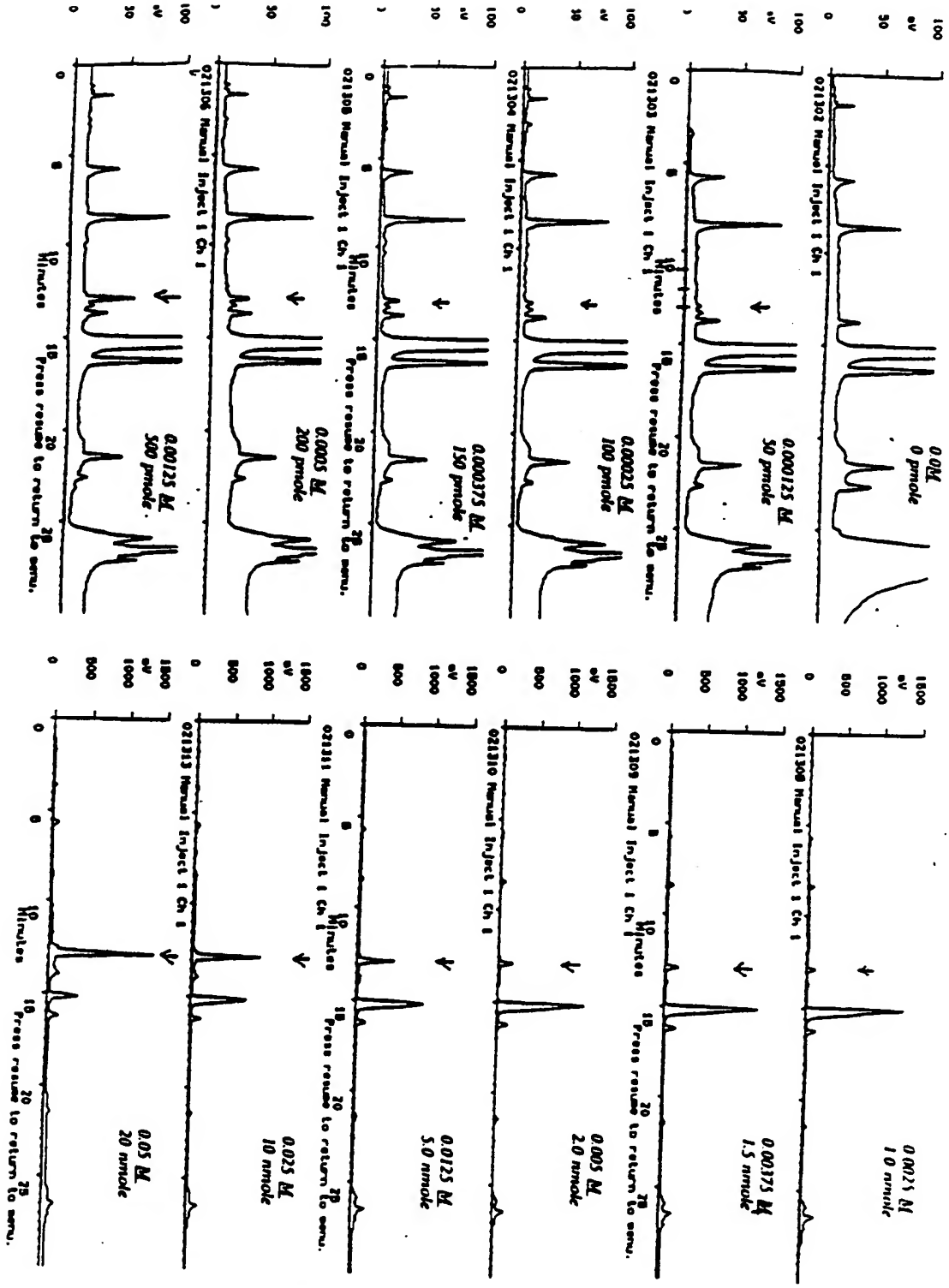
Derivatization of Glucose with Lucifer Yellow CH Standard Curve A210



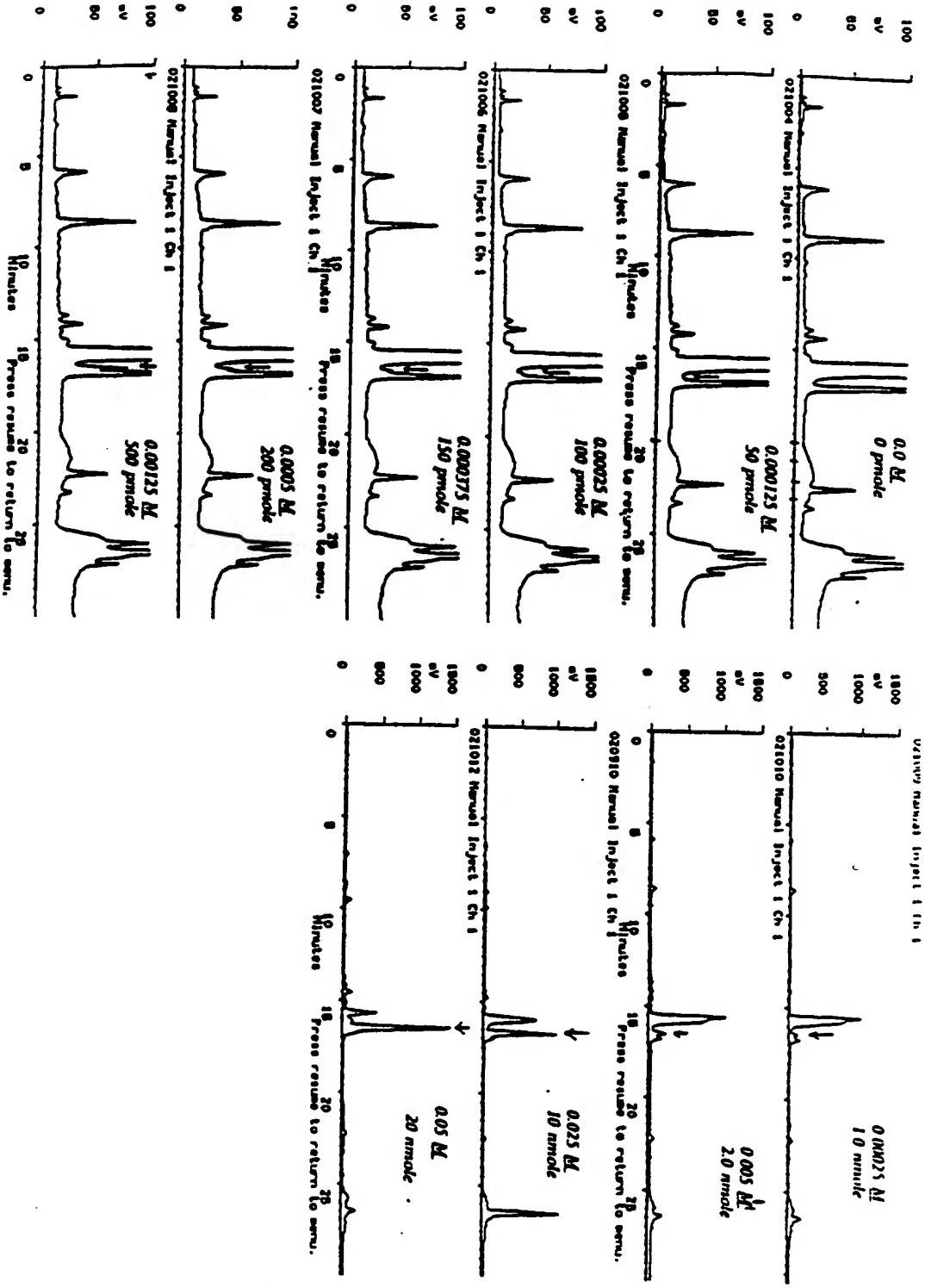
Derivatization of Galactose with Lucifer Yellow CH Standard Curve A210



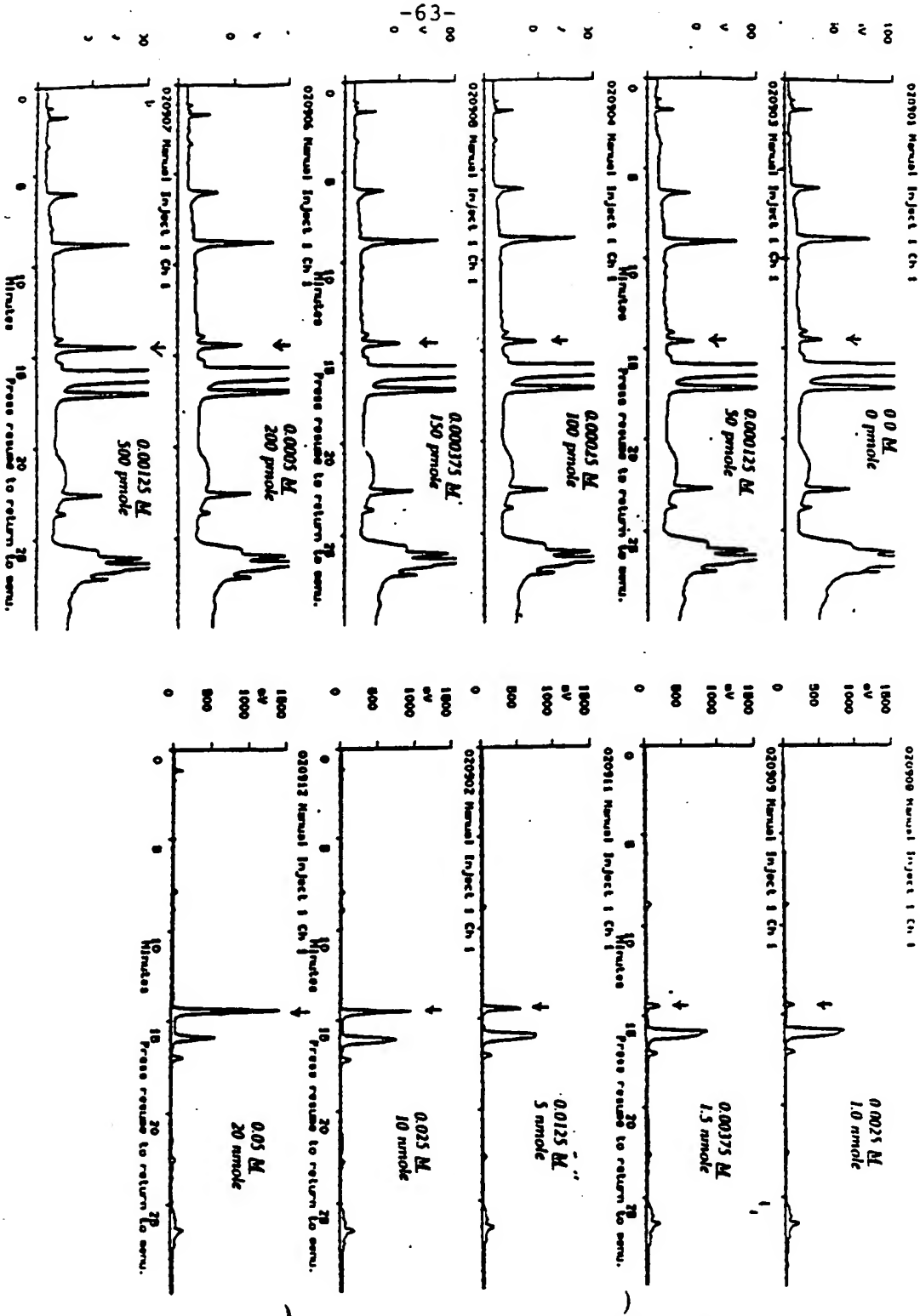
Derivatization of Galactose with Lucifer Yellow CH Standard Curve: Fluoresence, ex 430 nm, em cut-off 500 nm



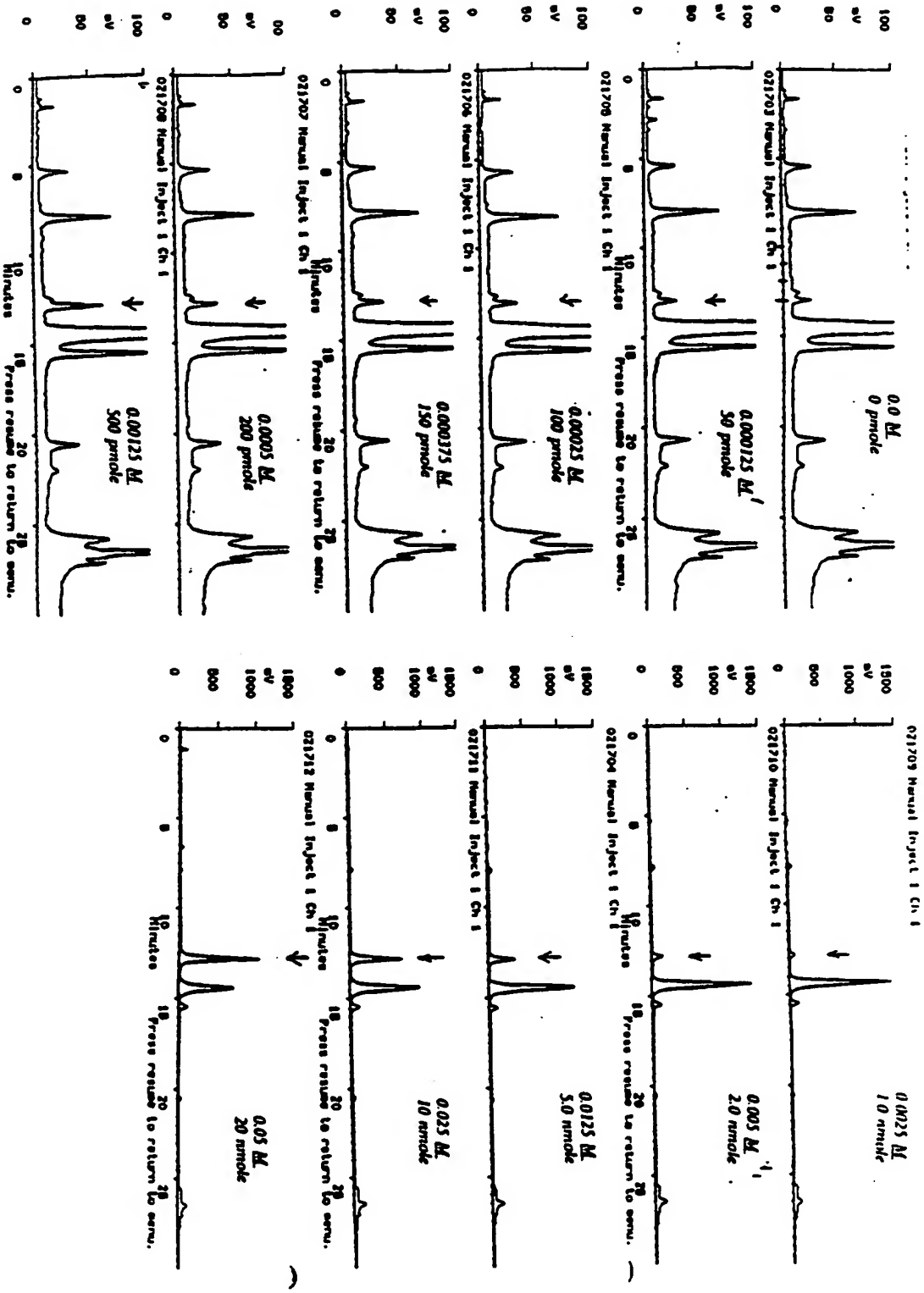
Derivatization of Mannose with Lucifer Yellow CH Standard Curve A210



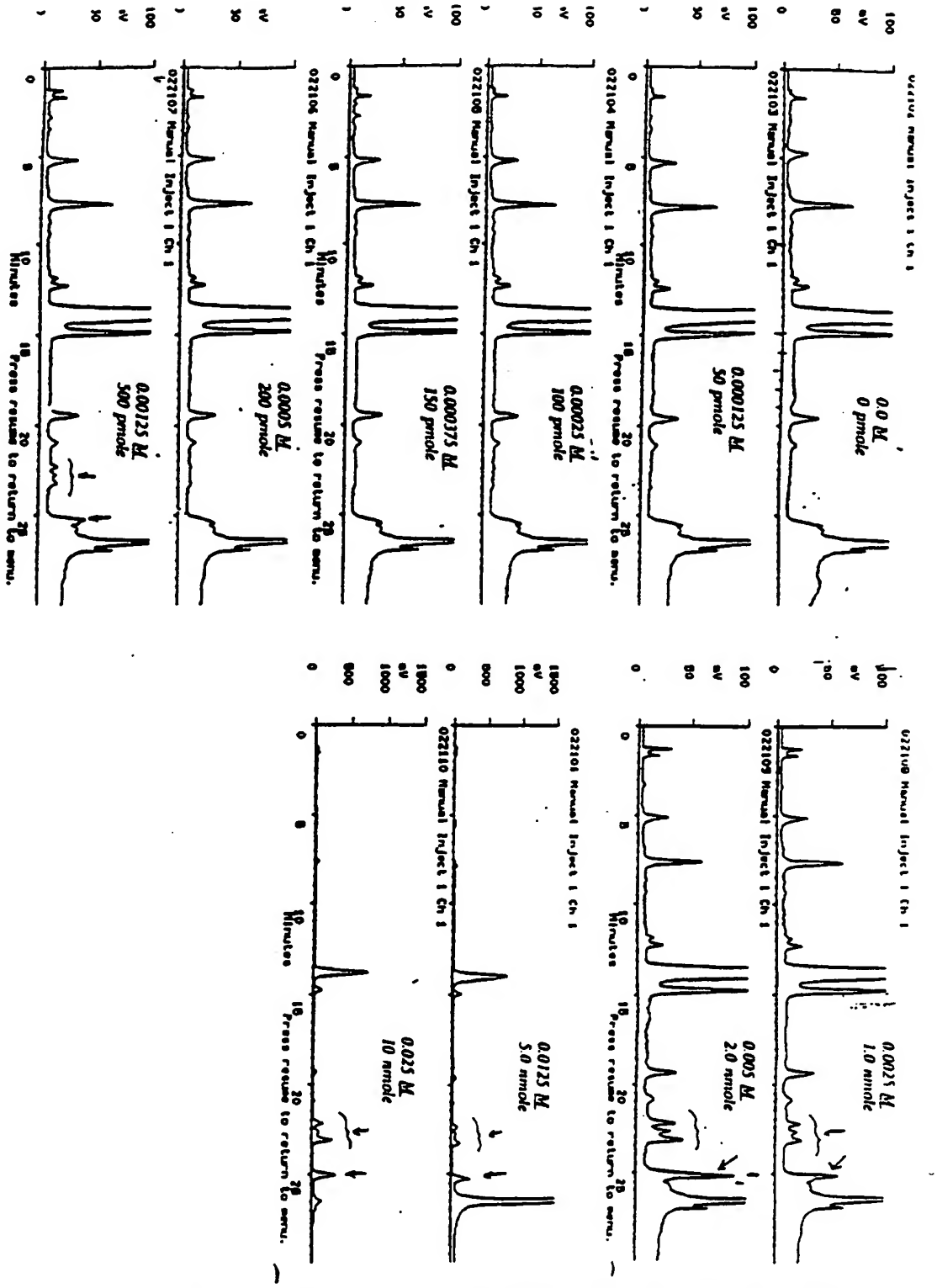
Derivatization of Fucose with Lucifer Yellow CH Standard Curve A210



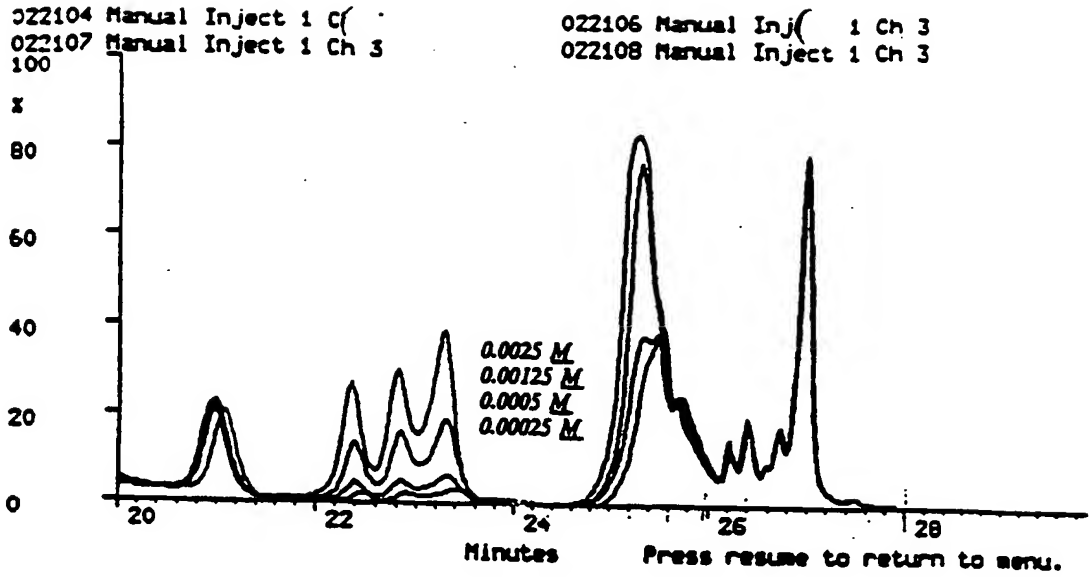
Derivatization of N-acetyl Glucosamine with Lucifer Yellow CH Standard Curve A210



Derivatization of N-acetyl Galactosamine with Lucifer Yellow CH Standard Curve A210



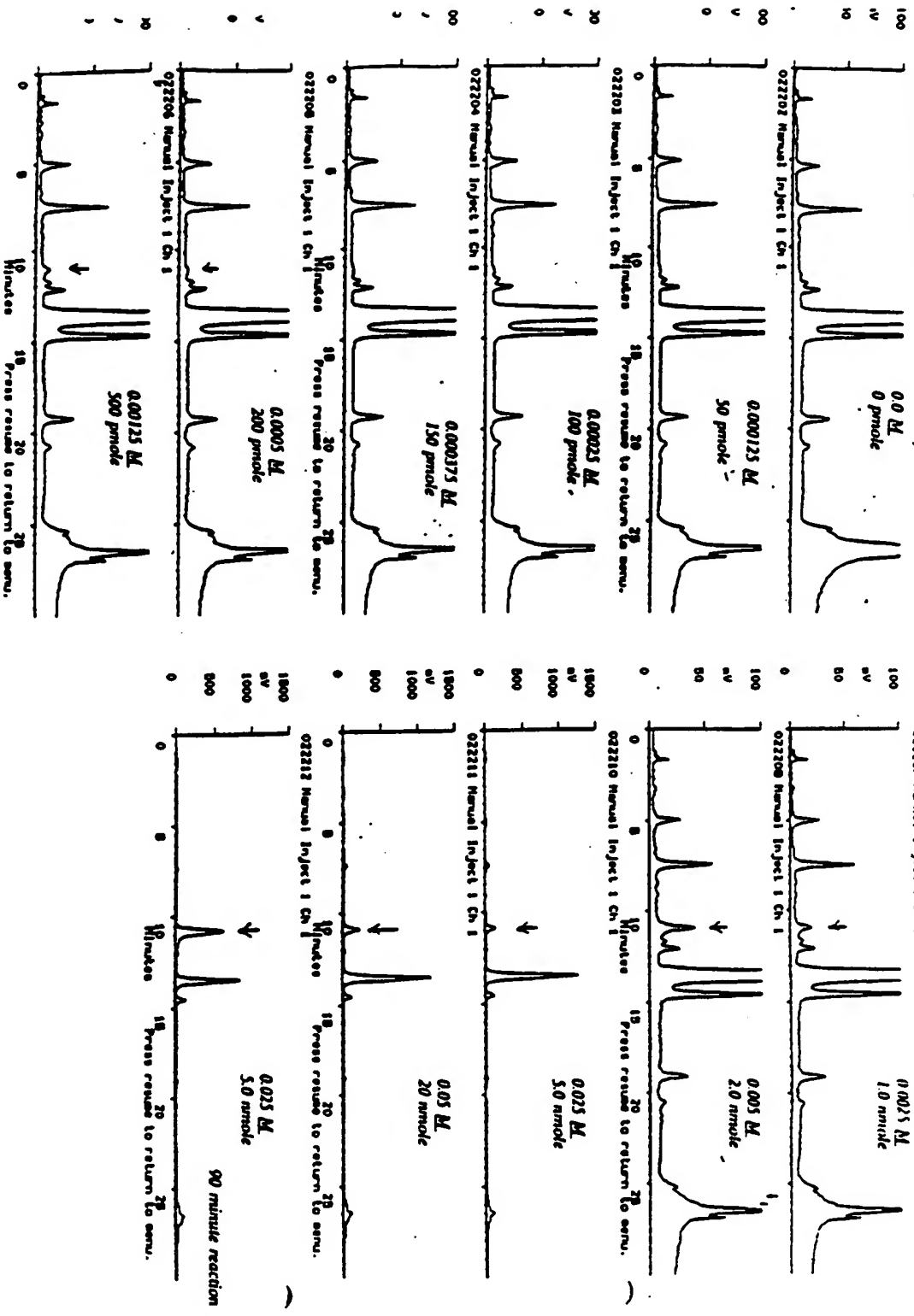
Derivatization of N-acetyl Neuraminic Acid with Lucifer Yellow CH Standard Curve A210



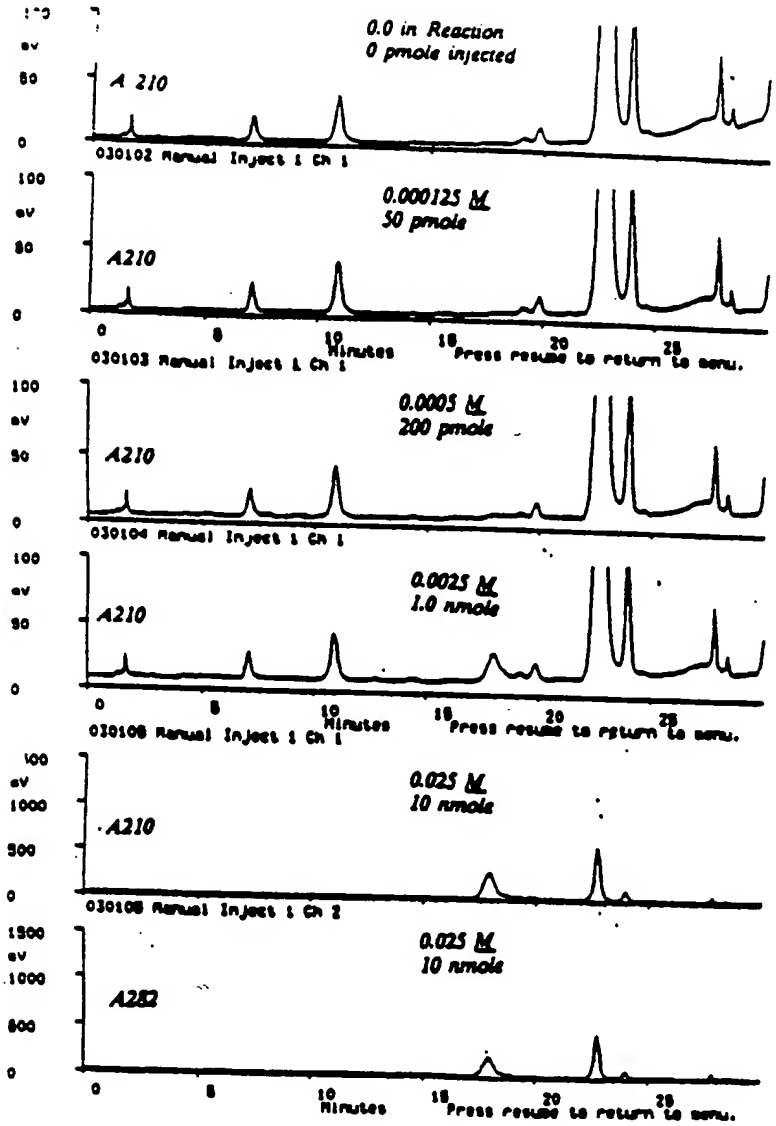
Derivatization of N-acetyl Neuraminic Acid with Lucifer Yellow CH

Fluorescence Detection
Ex 430 nm
Em 500 nm cut-off
Range 1 μA
y-axis 0-2000 mV

Concentration of sugar in reactions as indicated.

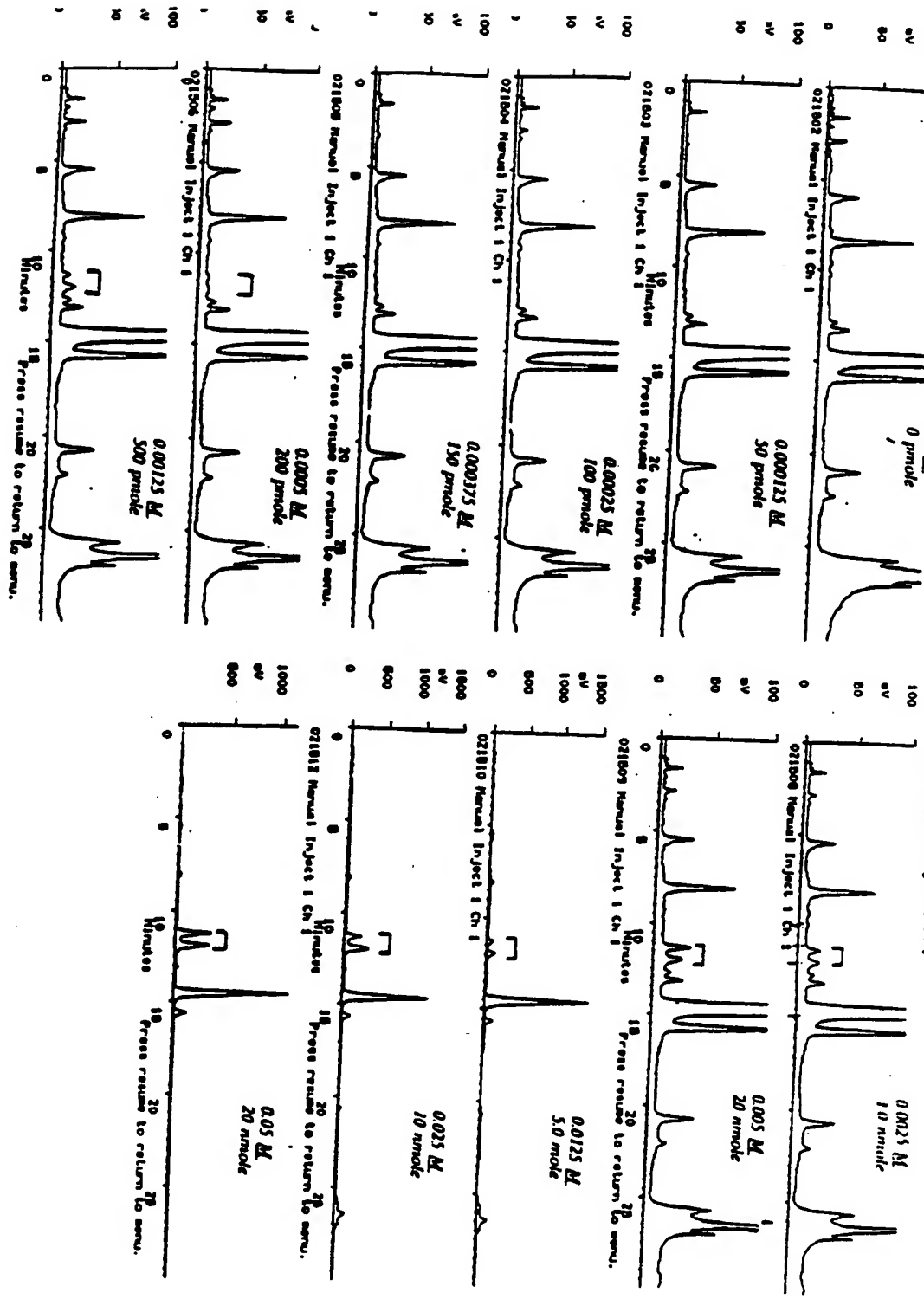


Derivatization of Glucosamine with Lucifer Yellow CH Standard Curve A210

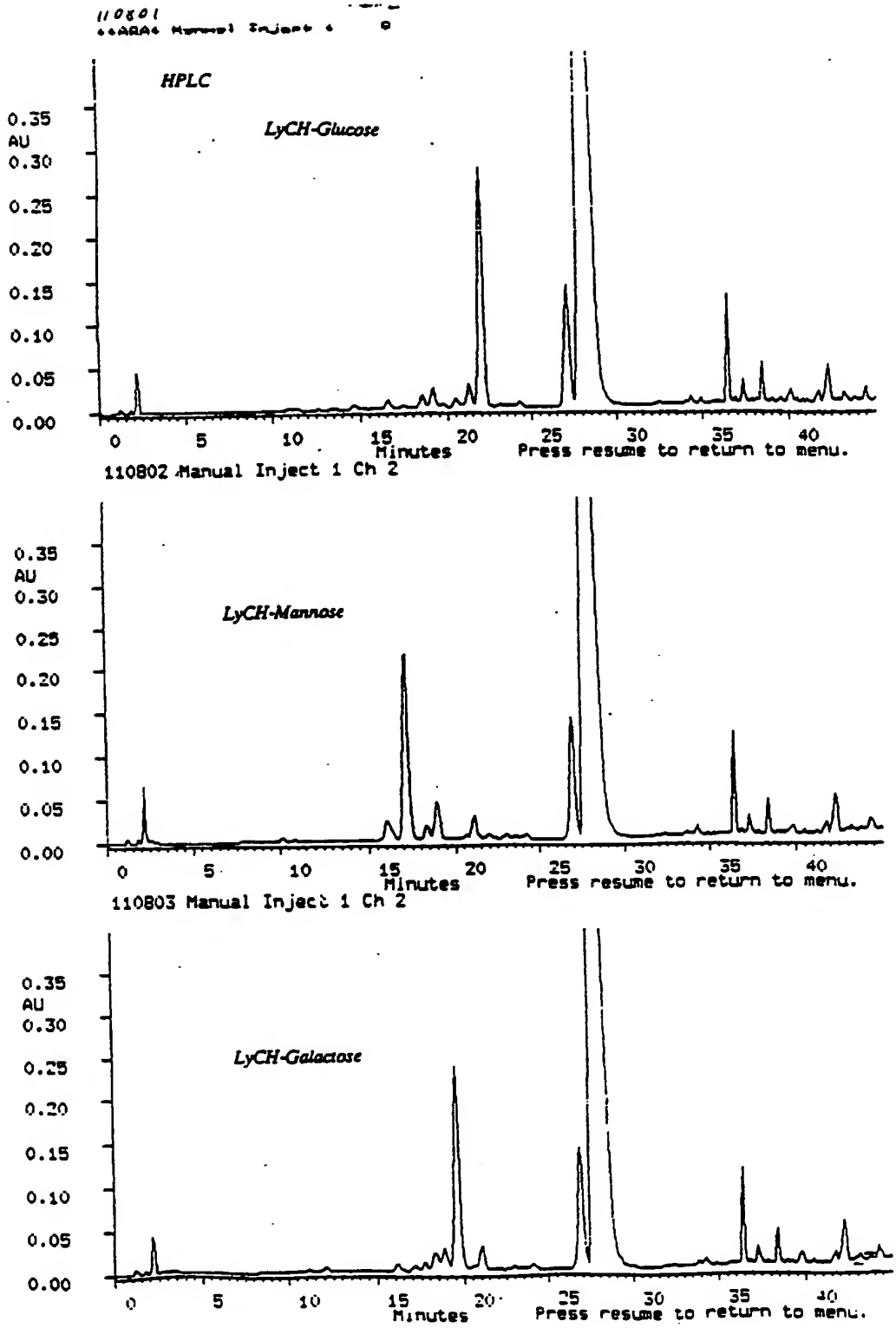


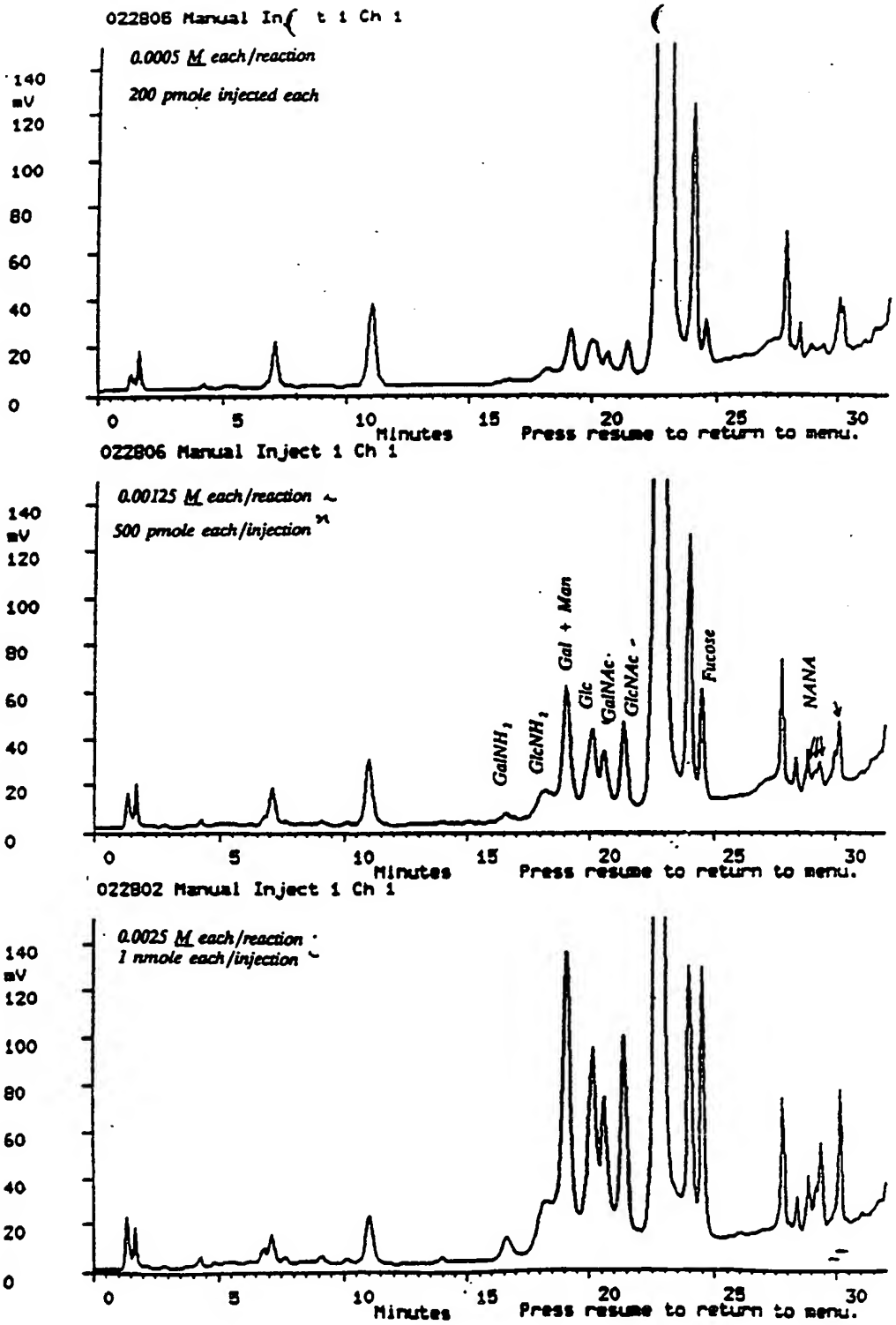
Gradient Program was that used for derivatized mixtures of monosaccharides.

Derivatization of Glucosamine with Lucifer Yellow CH 1.5 hr. Reaction Time



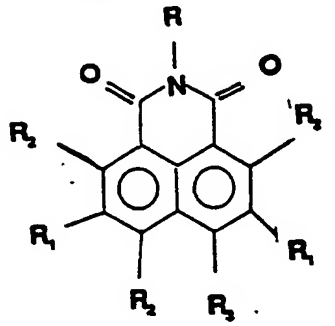
Derivatization of Galactosamine with Lucifer Yellow CH Standard Curve A210


HPLC
LyCH-Glucose
0.35
AU
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 5 10 15 20 25 30 35 40
Minutes
Press resume to return to menu.
110802 Manual Inject 1 Ch 2
LyCH-Mannose
110803 Manual Inject 1 Ch 2
LyCH-Galactose


022806 Manual Inject 1 Ch 1
0.0005 M each/reaction
200 pmole injected each
140
mV
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Minutes
Press resume to return to menu.
022806 Manual Inject 1 Ch 1
0.00125 M each/reaction
500 pmole each/injection
GalNH2
GlcNH2
Gal + Man
Glc
GalNAc
GlcNAc
Fucose
NANA
022802 Manual Inject 1 Ch 1
0.0025 M each/reaction
1 nmole each/injection

CLAIMS:

1. A method for preparing a derivatized saccharide including the step of reacting the saccharide with a compound having the formula:



or salts thereof, wherein R is $NHCONHNH_2$ or $CH_2(CH_2)_n NH_2$, R_1 is CO_2^-, OPO_3^- or SO_3^-; R_2 is hydrogen, a halide or a methyl group, and R_3 is NH_2 or H, and wherein n is 2, 3 or 4 when R is $CH_2(CH_2)_n NH_2$.

2. The method of claim 1, wherein R is $NHCONHNH_2$, R_1 is SO_3, R_2 is H and R_3 is NH_2.

3. The method of claim 1 wherein the method includes the additional step of separating the derivatized saccharide.

4. The method of claim 2, wherein the compound with which the saccharide is reacted is Lucifer Yellow CH.

5. The method of claim 3, wherein said derivatized saccharide is separated using at least on of gas

chromatography, liquid chromatography, high pressure liquid chromatography, paper chromatography, thin layer chromatography, and electrophoresis.

6. The method of claim 3, wherein the method includes the additional step of identifying the derivatized saccharide.

7. The method of claim 3, wherein the derivatized saccharide is identified using at least one of NMR, fluorescence spectroscopy, and mass spectrometry.

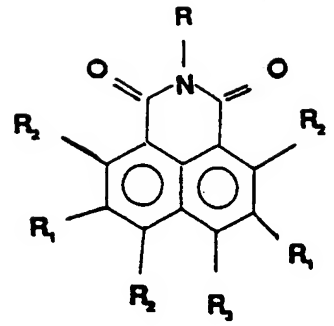
8. The method of claim 1, wherein the compound is an aminonapthylimide dye containing an R group capable of covalently interacting with a saccharide reducing end.

9. The method of claim 1, wherein the saccharide comprises a monosaccharide, disaccharide, polysaccharide or oligosaccharide.

10. The method of claim 1, wherein the saccharide comprises an amino-saccharide or N-acetylated aminosaccharide.

11. A method for identifying a saccharide including the steps of:

r acting in an acidic medium the saccharide with a compound having the formula:



or salts thereof, where R is $NHCONHNH_2$ or $CH_2(CH_2)_nNH_2$; R_1 is CO_2^-, OPO_3^- or SO_3^-; R_2 is hydrogen, a halide or a methyl group; R_3 is NH_2 or H; and wherein n is 2, 3 or 4 when R is $CH_2(CH_2)_n NH_2$;

separating the derivatized saccharide using one or more of gas chromatography, liquid chromatography, paper chromatography, thin layer chromatography, and electrophoresis; and

identifying the saccharide using HPLC, NMR, fluorescence spectroscopy or mass spectrometry.

12. The method of claim 11, wherein R is $NHCONHNH_2$, R_1 is SO_3^-, R_2 is hydrogen, and R_3 is NH_2.

13. The method of claim 11, wherein the saccharide comprises a monosaccharide, disaccharide, polysaccharide or oligosaccharide.

14. The method of claim 11, wherein the saccharide comprises a mixture of monosaccharides.

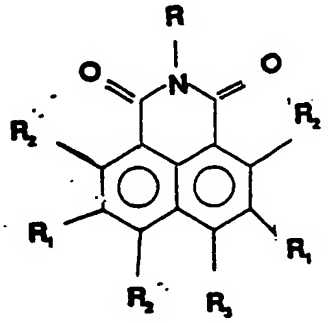
15. The method of claim 11, wherein the saccharide comprises a mixture of polysaccharides.

16. The method of claim 11, wherein the saccharide comprises an amino-saccharide or an N-acetylated saccharide.

17. A method for the identification of saccharides in a glycoprotein comprising the steps of:

separating the saccharide from the glycoprotein;

mixing the saccharide in the presence of an acid with a compound having the following structure:



or salts thereof, wherein R is $CH_2(CH_2)_nNH_2$ or $NHCONHNH_2$, R_1 is SO_3^-, CO_2^-, or OPO_3^-; R_2 is a hydrogen, a halide or a methyl group; R_3 is NH_2 or H; wherein n is 2, 3 or 4 when R is $CH_2(CH_2)_n NH_2$;

heating the reaction mixture for between 30 minutes to 180 minutes to form a mixture of derivatized saccharides;

separating the mixture of derivatized saccharides using one or more of gas chromatography, liquid chromatography, paper chromatography, then layer chromatography, and electrophoresis; and

identifying the saccharides in the glycoprotein using HPLC, NMR, fluorescence spectroscopy or mass spectrometry.

18. The method of claim 17, wherein R is $NHCONHNH_2$, R_1 is SO_3^-, R_2 is hydrogen, and R_3 is NH_2.

19. The method of claim 17, wherein the saccharides are a mixture of monosaccharides and the reaction mixture is heated about 90 minutes.

20. The method of claim 17, wherein the mixture of derivatized saccharides is separated by electrophoresis.

21. The method of claim 17, wherein the saccharides are identified by HPLC.

22. The method of claim 17, wherein the acid is acetic acid.

23. The method of claim 17, wherein separating the saccharides from the glycoprotein compris s hydr lyzing the glycoprotein to form a mixture of saccharides.

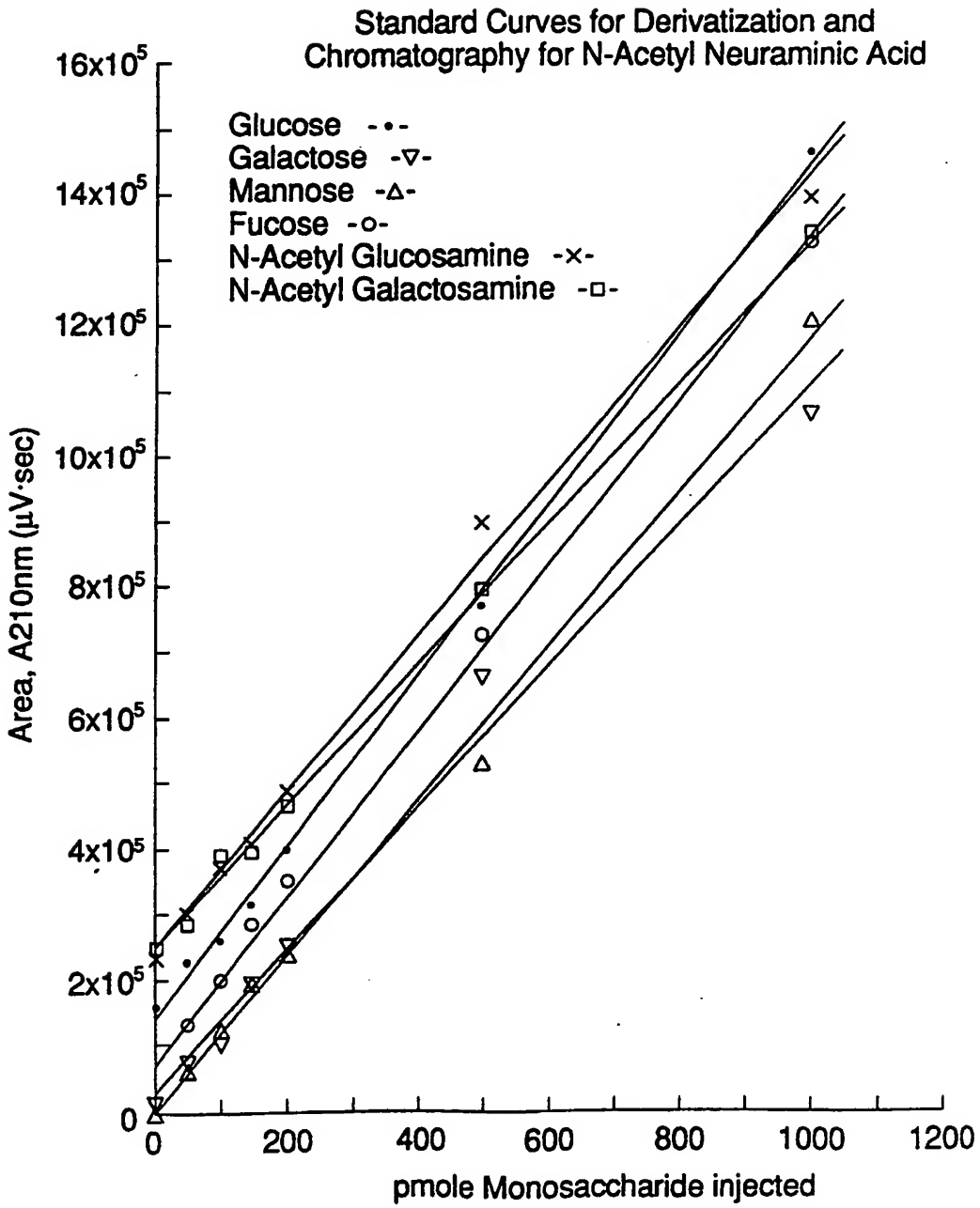

Standard Curves for Derivatization and
Chromatography for N-Acetyl Neuraminic Acid
Glucose -•-
Galactose -▽-
Mannose -△-
Fucose -○-
N-Acetyl Glucosamine -×-
N-Acetyl Galactosamine -□-
Area, A210nm (μV·sec)
16x10^5
14x10^5
12x10^5
10x10^5
8x10^5
6x10^5
4x10^5
2x10^5
0
0
200
400
600
800
1000
1200
pmole Monosaccharide injected

Fig. 1

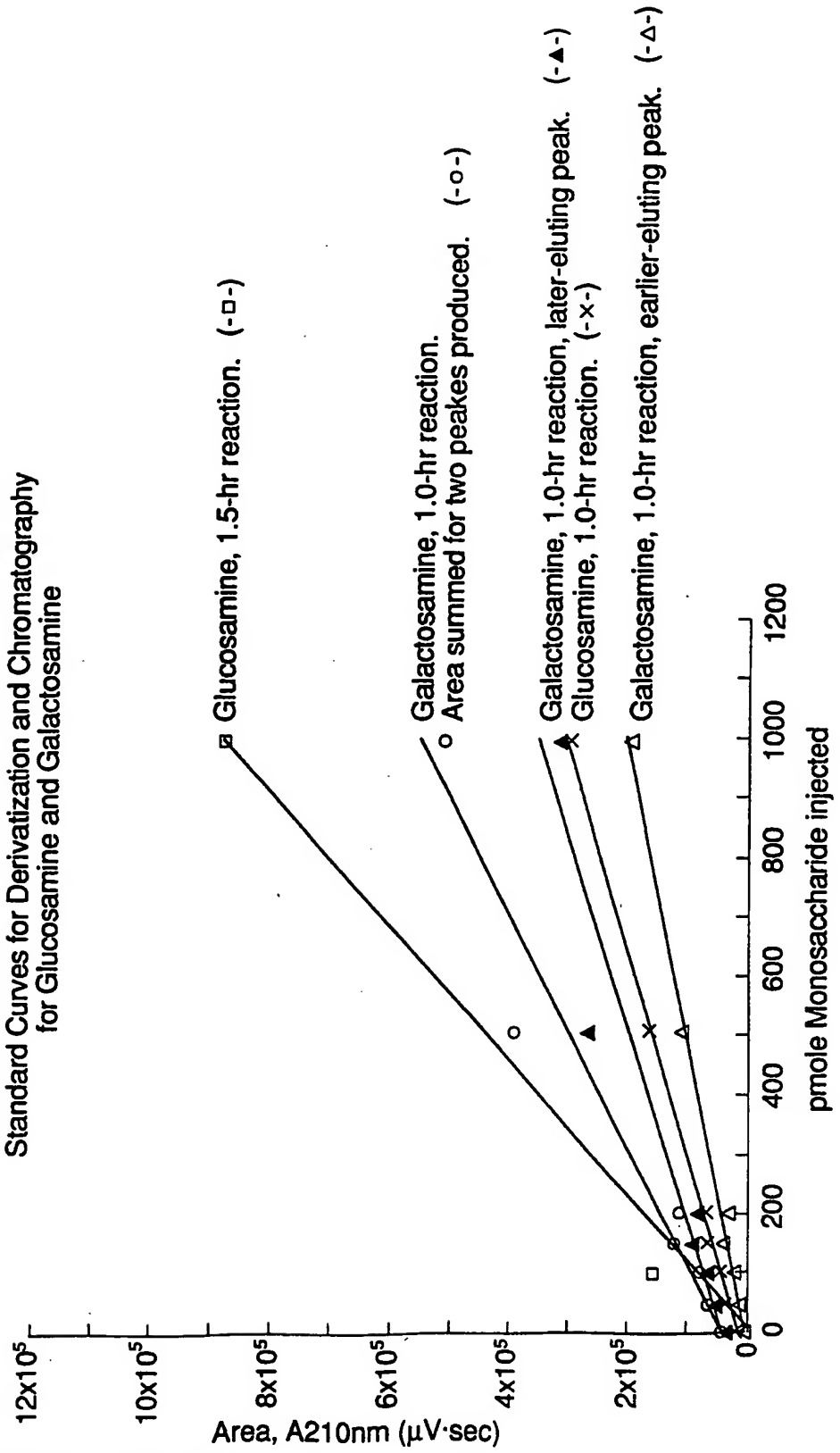

Standard Curves for Derivatization and Chromatography
for Glucosamine and Galactosamine
Glucosamine, 1.5-hr reaction. (-□-)
Galactosamine, 1.0-hr reaction.
Area summed for two peakes produced. (-○-)
Galactosamine, 1.0-hr reaction, later-eluting peak. (-▲-)
Glucosamine, 1.0-hr reaction. (-×-)
Galactosamine, 1.0-hr reaction, earlier-eluting peak. (-△-)
Area, A210nm (μV·sec)
12x10^5
10x10^5
8x10^5
6x10^5
4x10^5
2x10^5
0
0
200
400
600
800
1000
1200
pmole Monosaccharide injected

Fig. 2

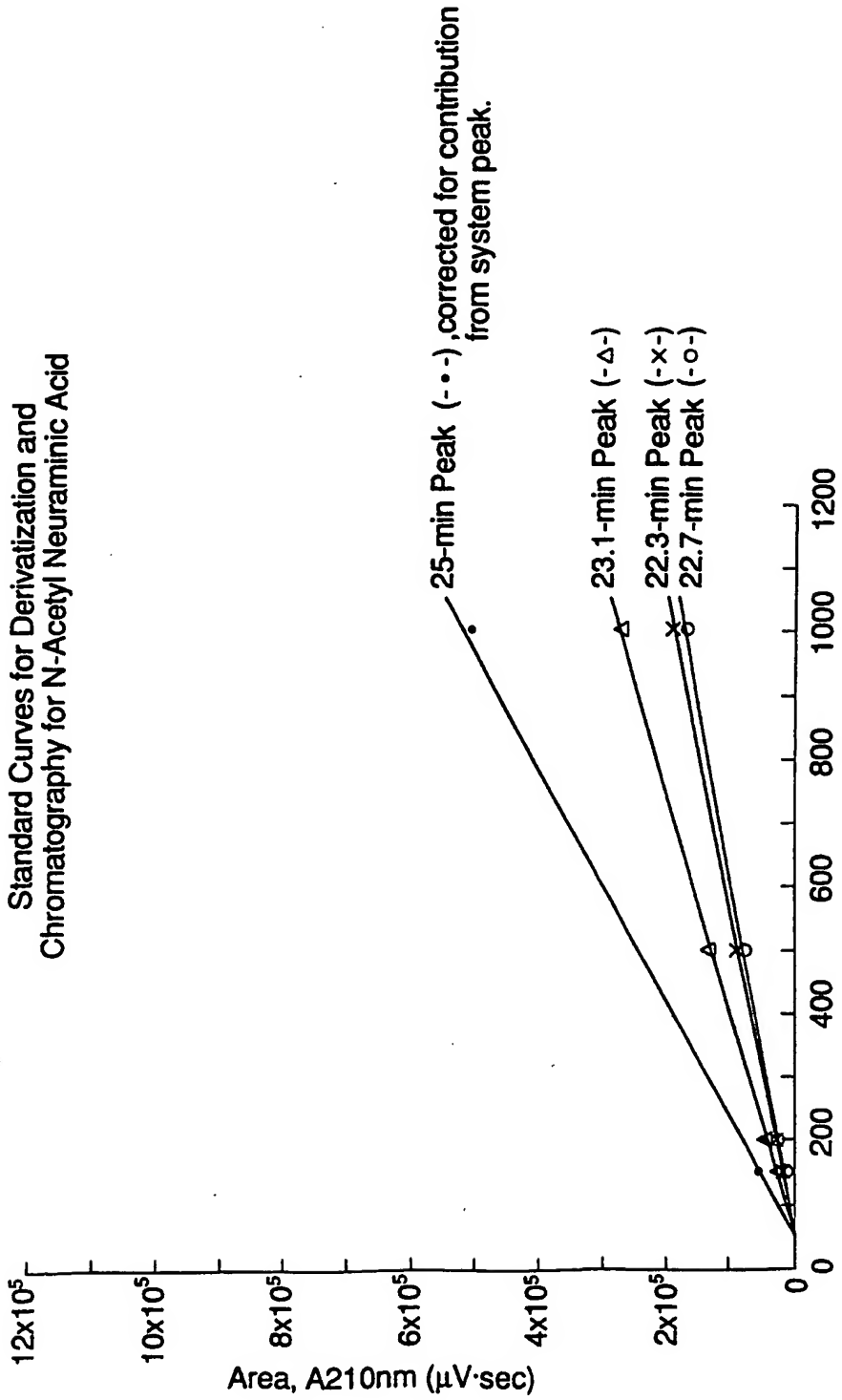

Standard Curves for Derivatization and Chromatography for N-Acetyl Neuraminic Acid
25-min Peak (-•-), corrected for contribution from system peak.
23.1-min Peak (-△-)
22.3-min Peak (-×-)
22.7-min Peak (-○-)
Area, A210nm (μV·sec)
12x10^5
10x10^5
8x10^5
6x10^5
4x10^5
2x10^5
0
0
200
400
600
800
1000
1200
pmole Monosaccharide injected

Fig. 3

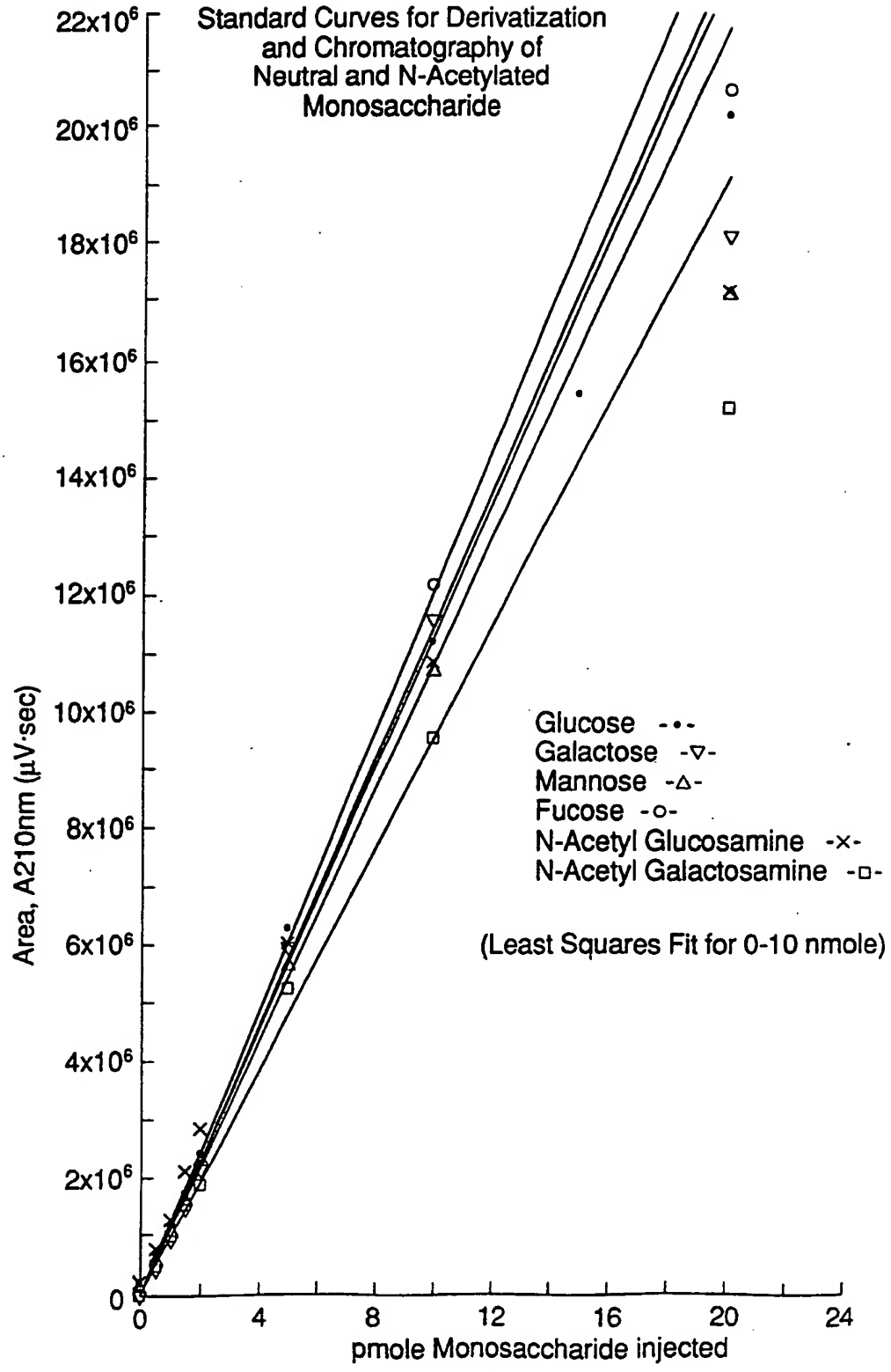

Standard Curves for Derivatization and Chromatography of Neutral and N-Acetylated Monosaccharide
Area, A210nm (μV·sec)
22x10⁶
20x10⁶
18x10⁶
16x10⁶
14x10⁶
12x10⁶
10x10⁶
8x10⁶
6x10⁶
4x10⁶
2x10⁶
0
0
4
8
12
16
20
24
pmole Monosaccharide injected
Glucose -•-
Galactose -▽-
Mannose -△-
Fucose -○-
N-Acetyl Glucosamine -×-
N-Acetyl Galactosamine -□-
(Least Squares Fit for 0-10 nmole)

Fig. 4

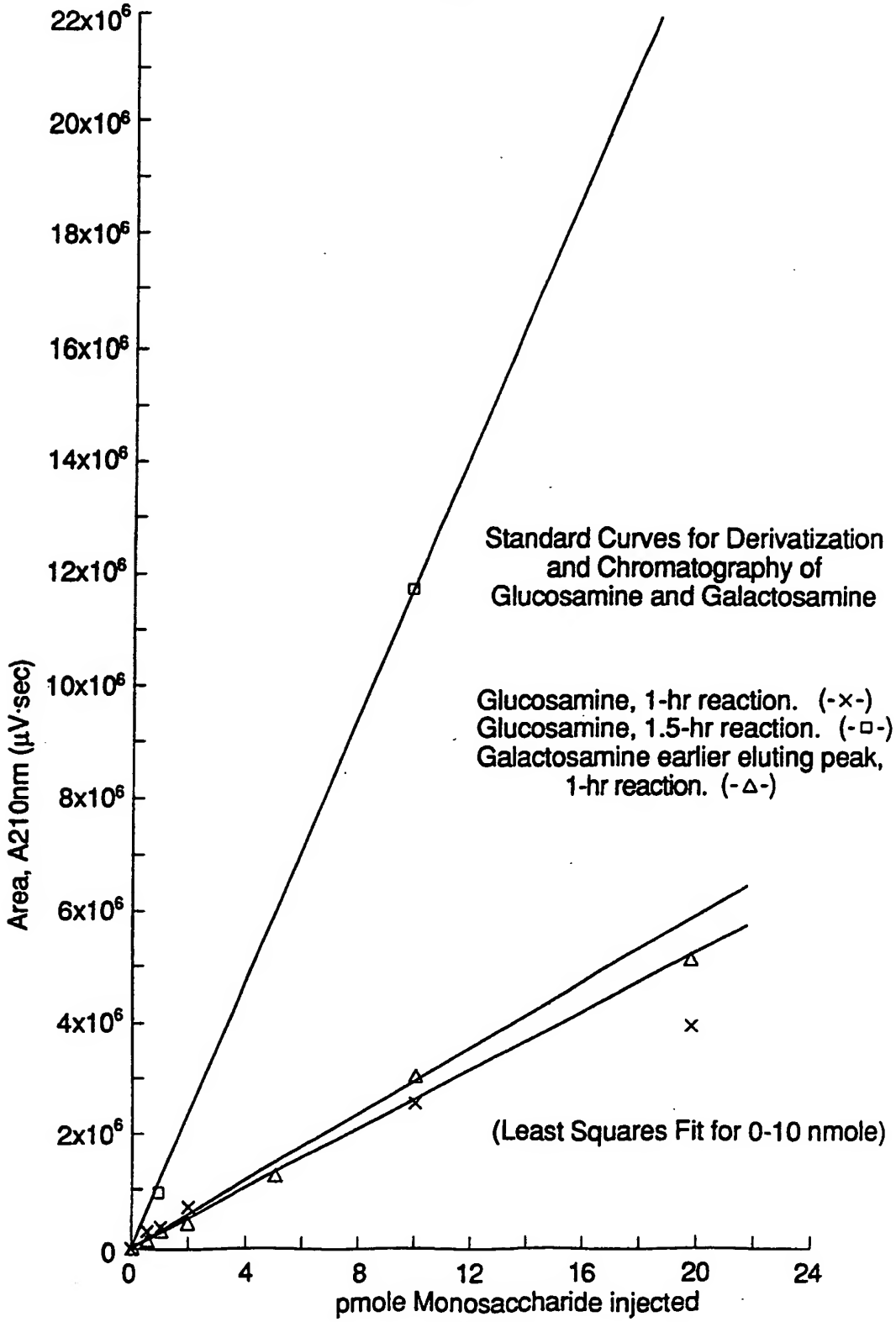

22x10⁶
20x10⁶
18x10⁶
16x10⁶
14x10⁶
12x10⁶
10x10⁶
8x10⁶
6x10⁶
4x10⁶
2x10⁶
0
Area, A210nm (μV·sec)
0
4
8
12
16
20
24
pmole Monosaccharide injected
Standard Curves for Derivatization and Chromatography of Glucosamine and Galactosamine
Glucosamine, 1-hr reaction. (-×-)
Glucosamine, 1.5-hr reaction. (-□-)
Galactosamine earlier eluting peak, 1-hr reaction. (-△-)
(Least Squares Fit for 0-10 nmole)

Fig. 5

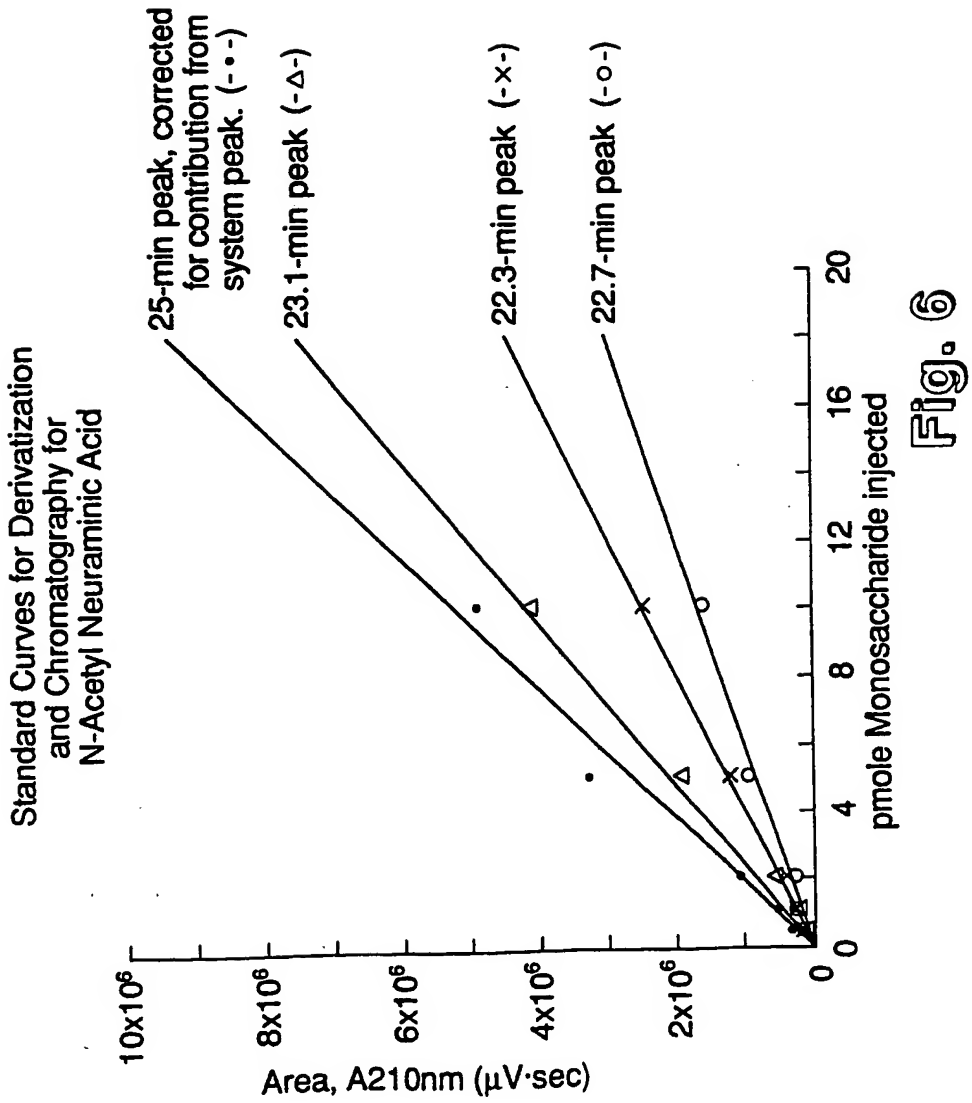

Standard Curves for Derivatization and Chromatography for N-Acetyl Neuraminic Acid
25-min peak, corrected for contribution from system peak. (-•-)
23.1-min peak (-Δ-)
22.3-min peak (-x-)
22.7-min peak (-o-)
Area, A210nm (μV·sec)
10x10^6
8x10^6
6x10^6
4x10^6
2x10^6
0
0
4
8
12
16
20
pmole Monosaccharide injected

Fig. 6

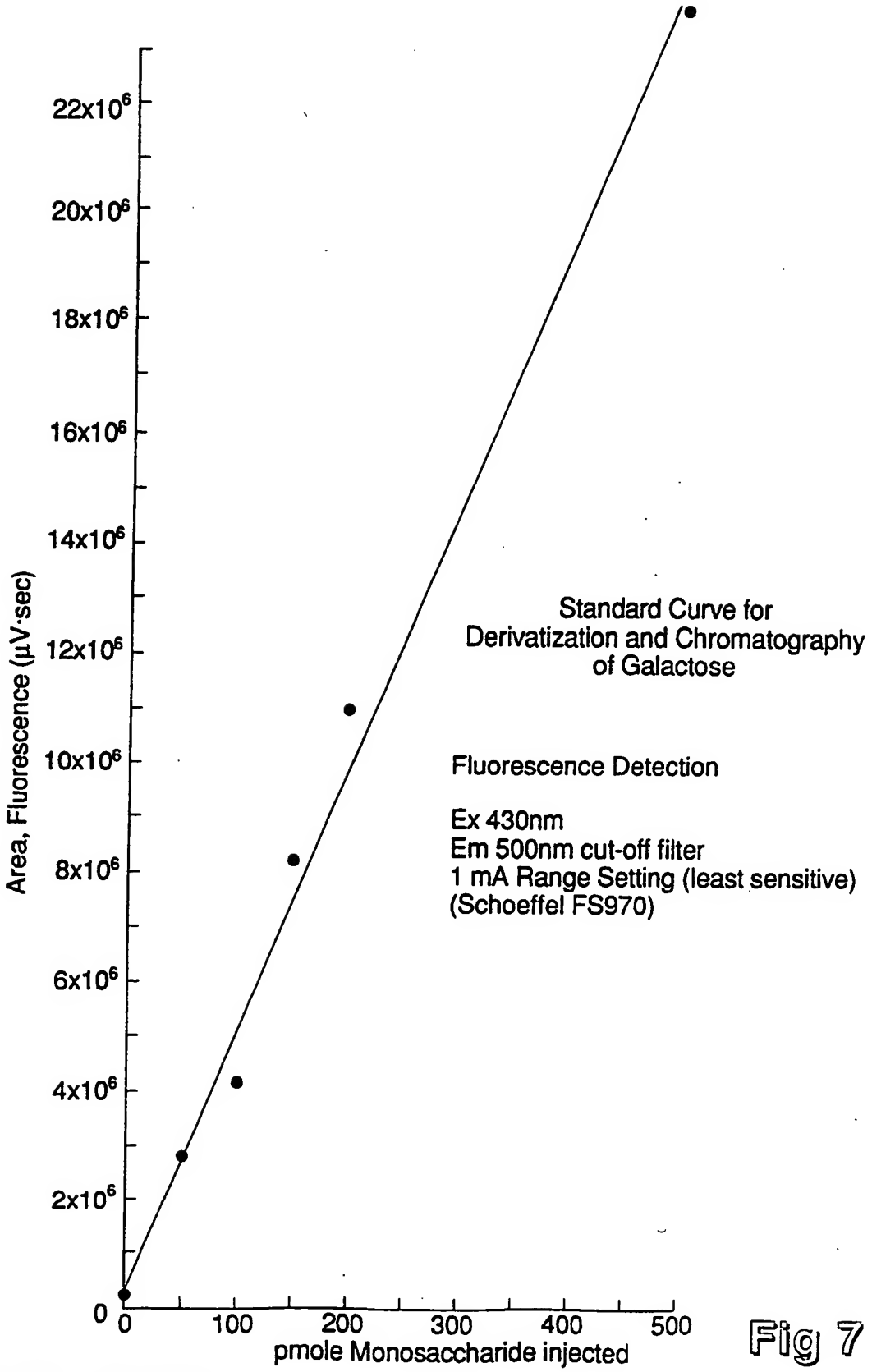
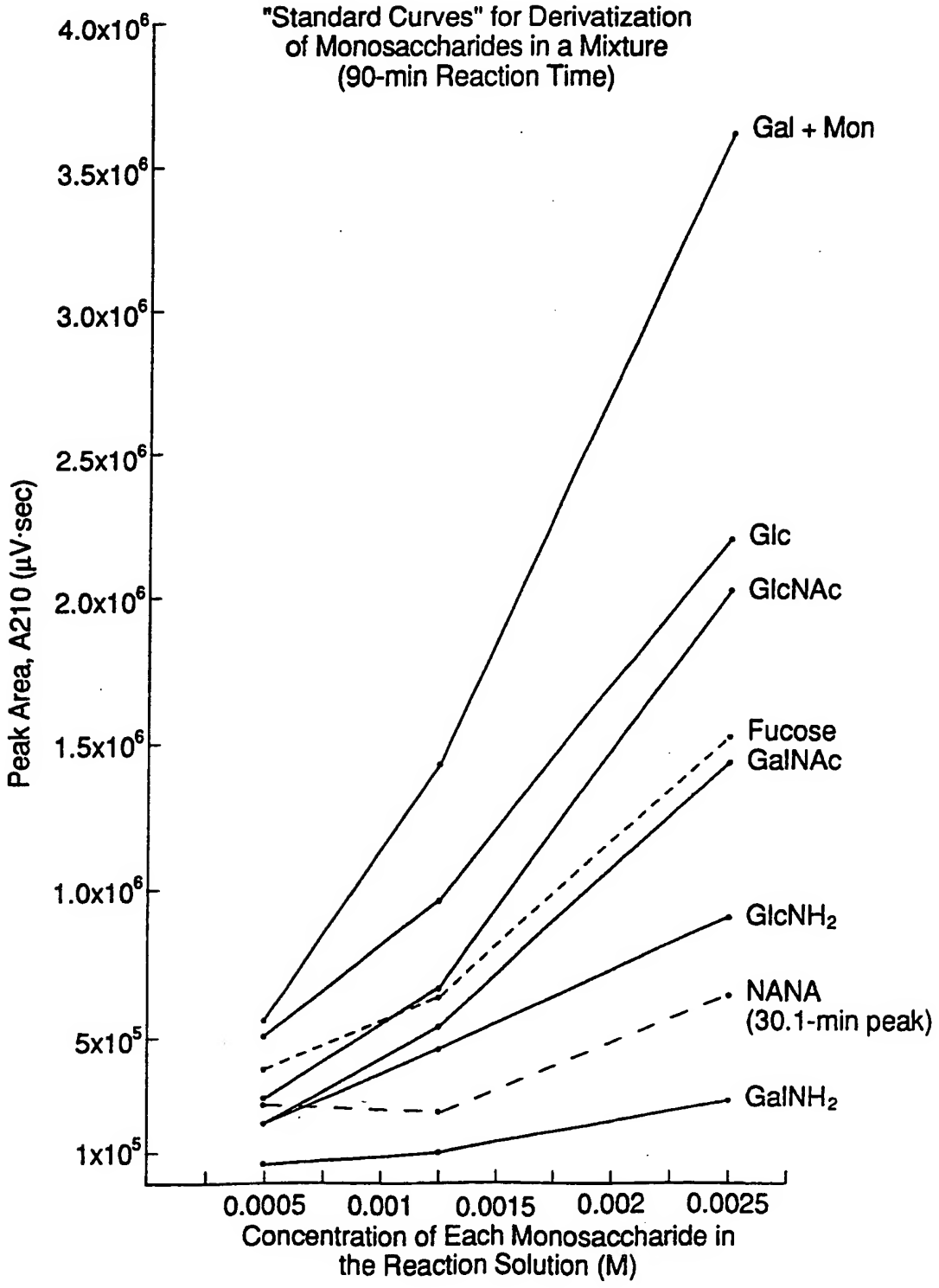

22x10⁶
20x10⁶
18x10⁶
16x10⁶
14x10⁶
12x10⁶
10x10⁶
8x10⁶
6x10⁶
4x10⁶
2x10⁶
0
Area, Fluorescence (μV·sec)
0
100
200
300
400
500
pmole Monosaccharide injected
Standard Curve for
Derivatization and Chromatography
of Galactose
Fluorescence Detection
Ex 430nm
Em 500nm cut-off filter
1 mA Range Setting (least sensitive)
(Schoeffel FS970)

Fig 7



SUBSTITUTE SHEET

Fig. 8

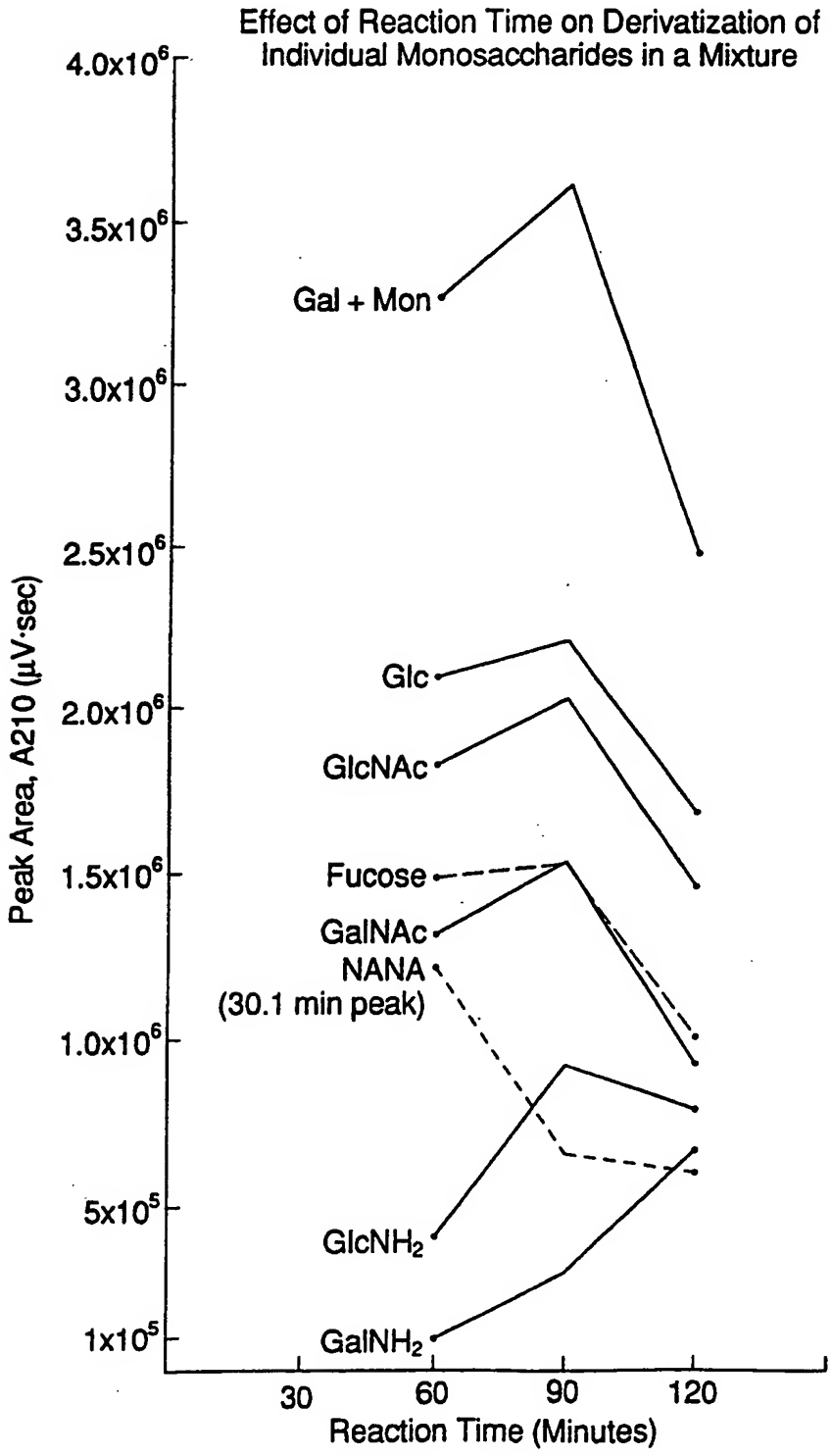

Effect of Reaction Time on Derivatization of Individual Monosaccharides in a Mixture
4.0x10^6
3.5x10^6
3.0x10^6
2.5x10^6
2.0x10^6
1.5x10^6
1.0x10^6
5x10^5
1x10^5
Peak Area, A210 (μV·sec)
Gal + Mon
Glc
GlcNAc
Fucose
GalNAc
NANA
(30.1 min peak)
GlcNH2
GalNH2
30
60
90
120
Reaction Time (Minutes)

Fig. 9

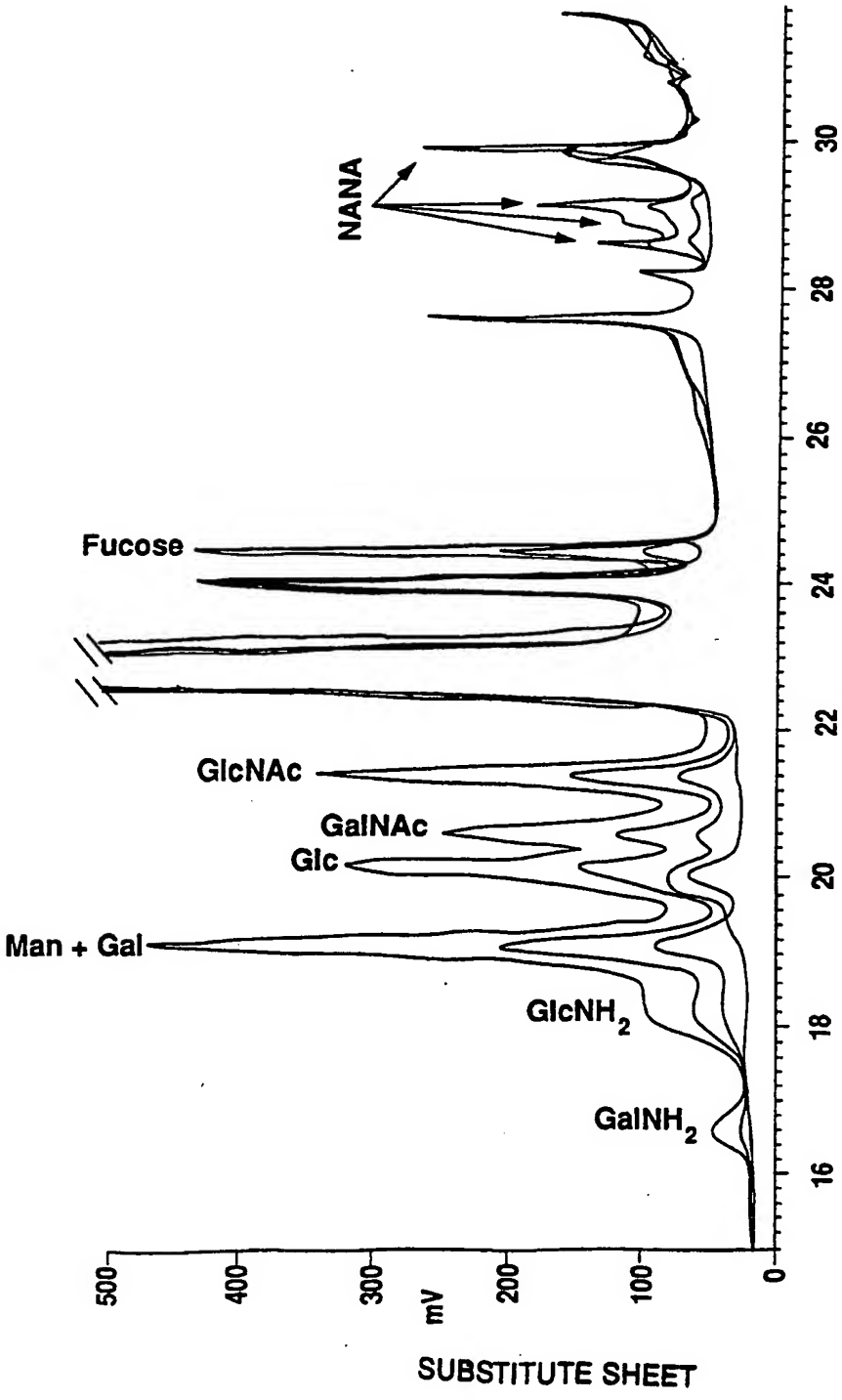

NANA
Fucose
GlcNAc
GalNAc
Glc
Man + Gal
GlcNH2
GalNH2
16
18
20
22
24
26
28
30
MINUTES
0
100
200
300
400
500
mV

Fig. 10

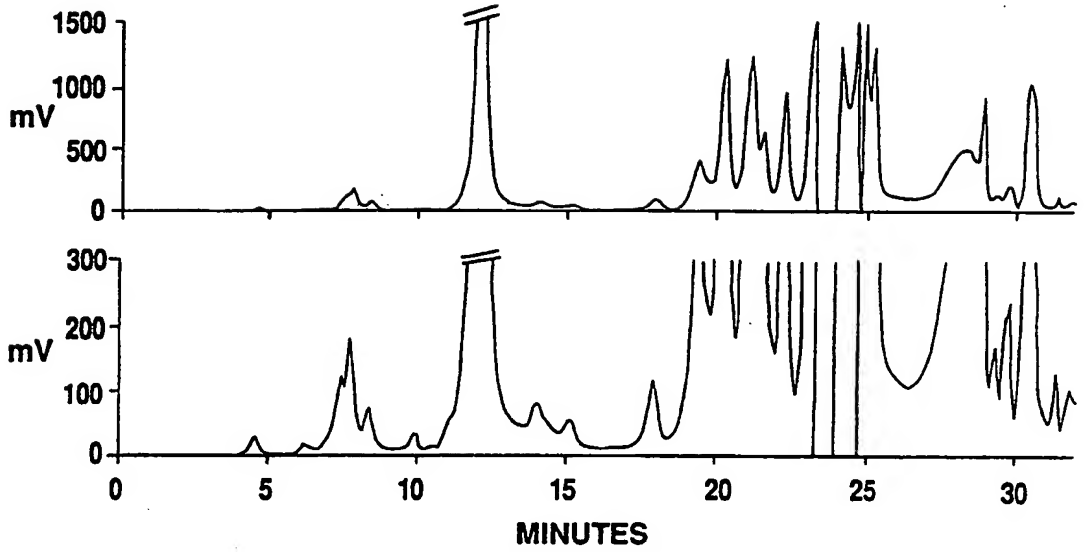


Fig. 11A

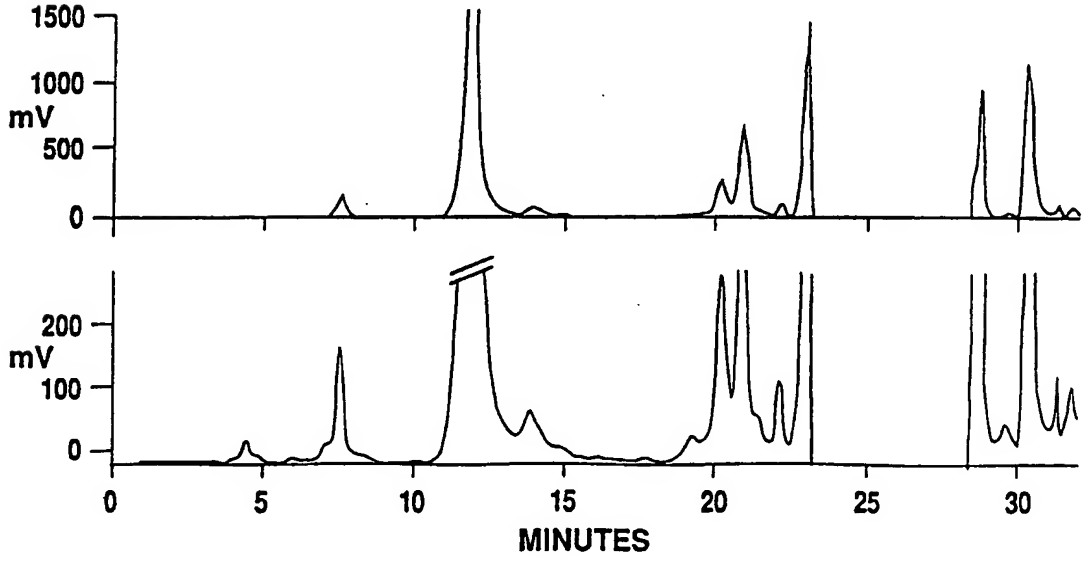


Fig. 11B

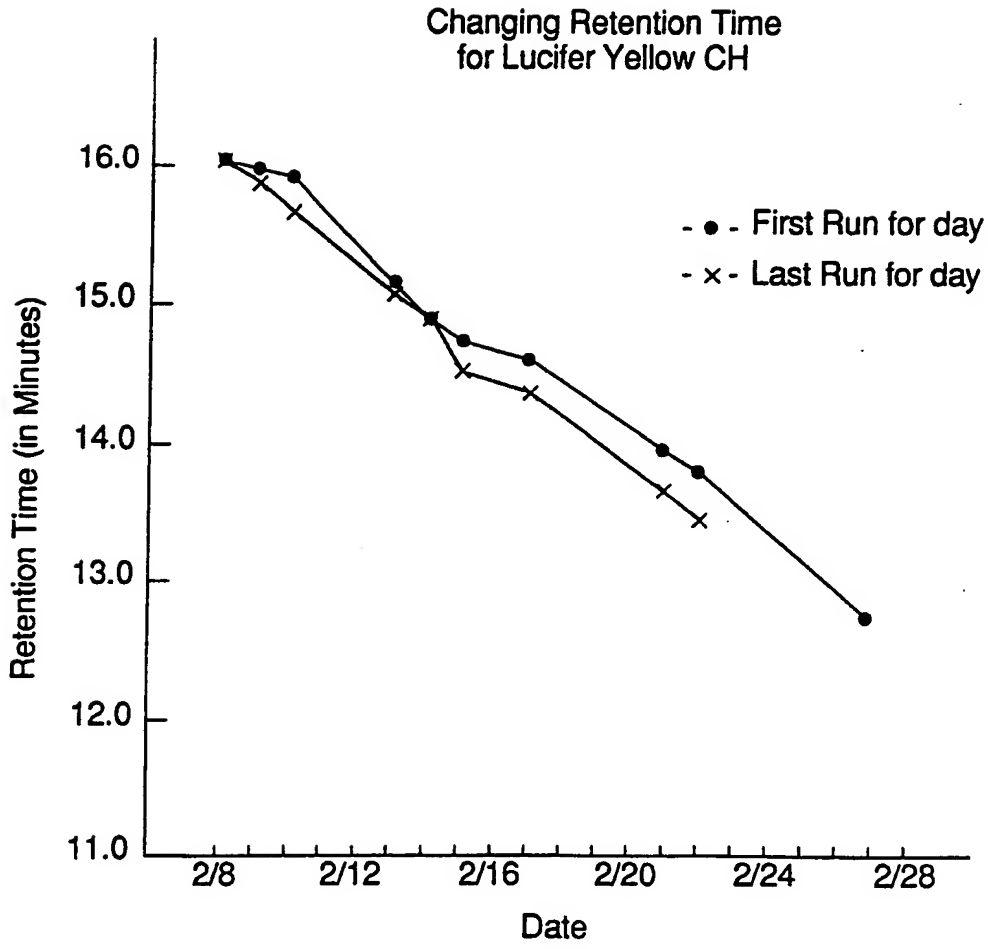

Changing Retention Time
for Lucifer Yellow CH
16.0
15.0
14.0
13.0
12.0
11.0
Retention Time (in Minutes)
- ● - First Run for day
- × - Last Run for day
2/8
2/12
2/16
2/20
2/24
2/28
Date

Fig. 12

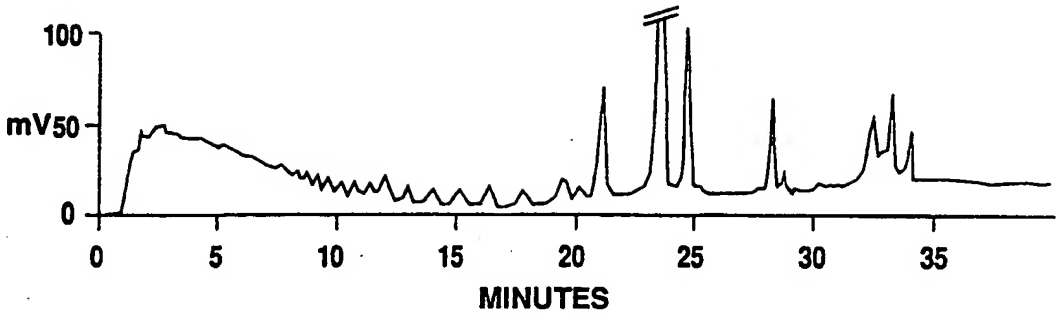


Fig. 13A

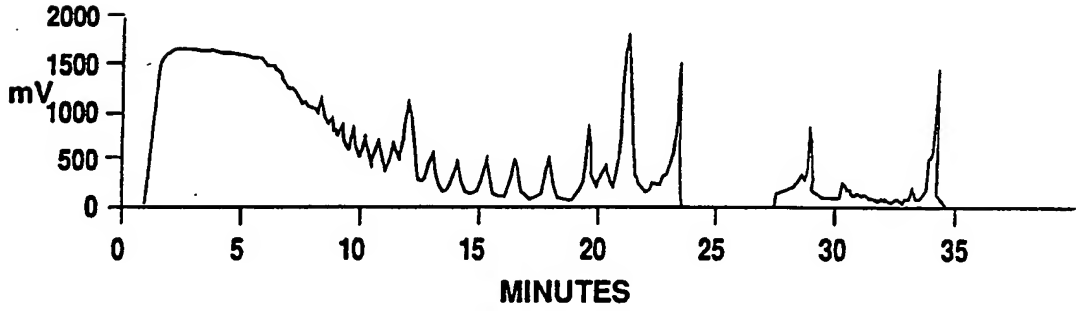


Fig. 13B

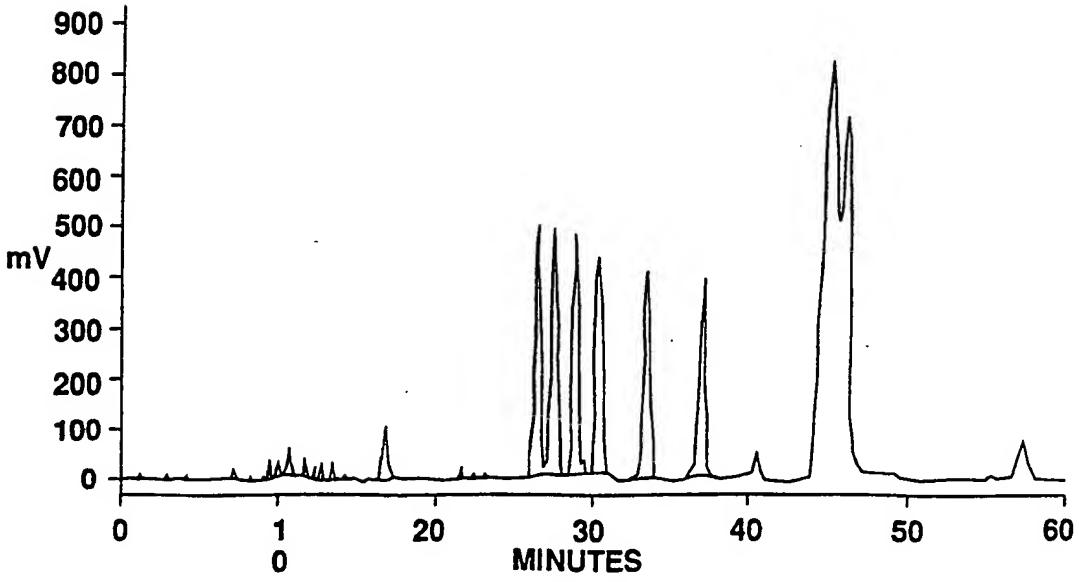


Fig. 14

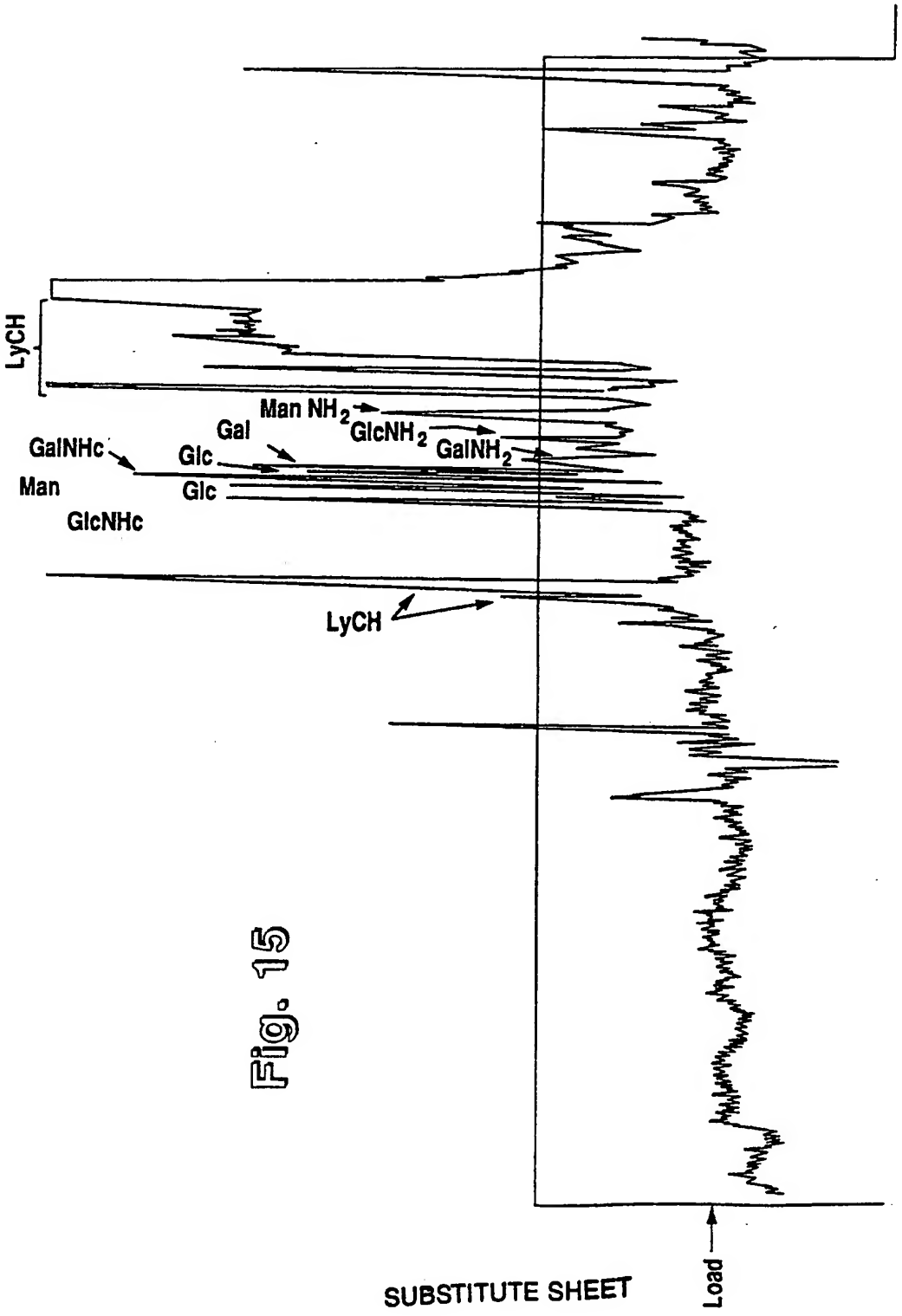

LyCH
Man NH2
Gal
GlcNH2
GalNHc
Glc
GalNH2
Man
Glc
GlcNHc
LyCH
Load

Fig. 15

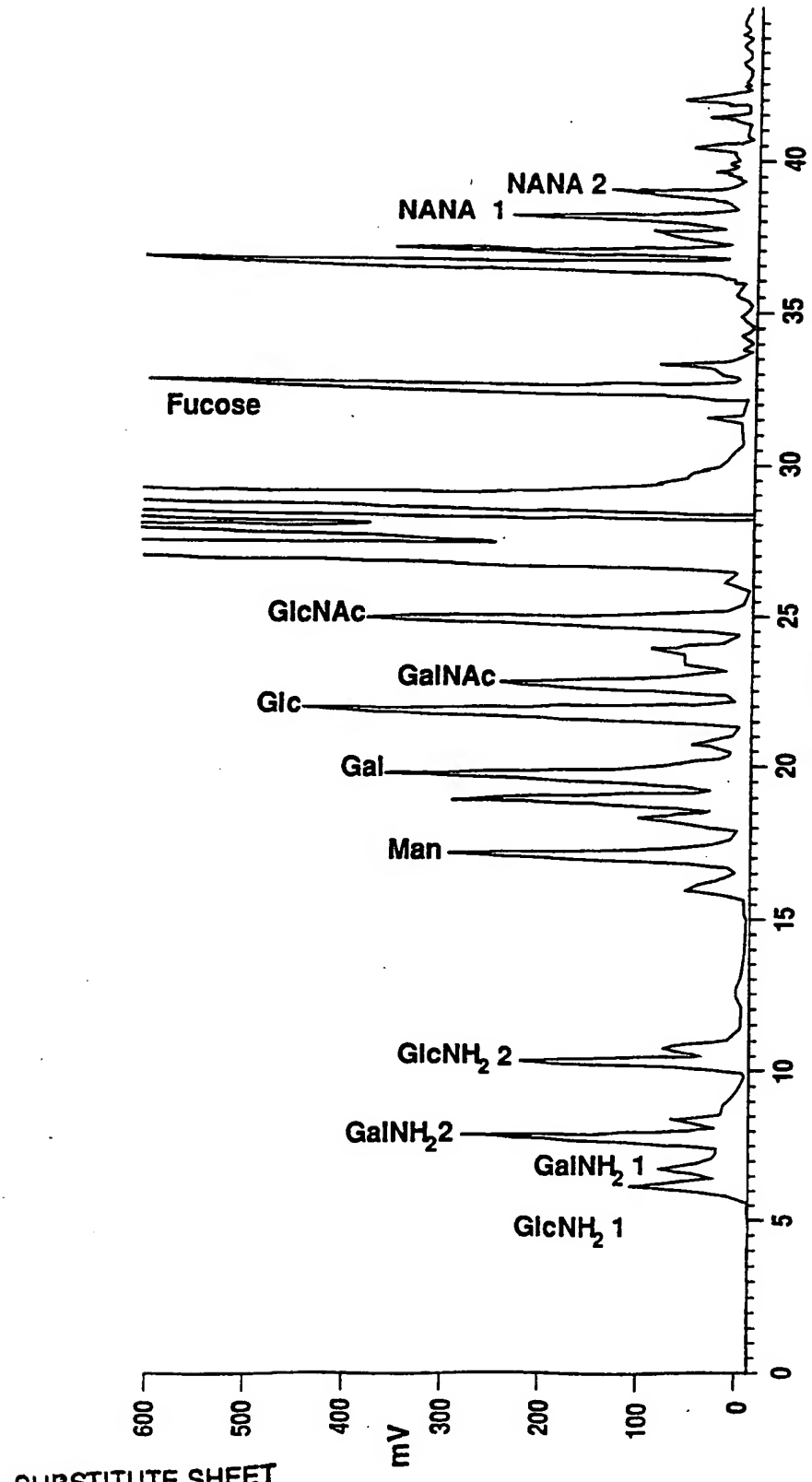

NANA 2
NANA 1
Fucose
GlcNAc
GalNAc
Glc
Gal
Man
GlcNH2 2
GalNH2 2
GalNH2 1
GlcNH2 1
0
5
10
15
20
25
30
35
40
MINUTES
600
500
400
300
200
100
0
mV

Fig. 16A

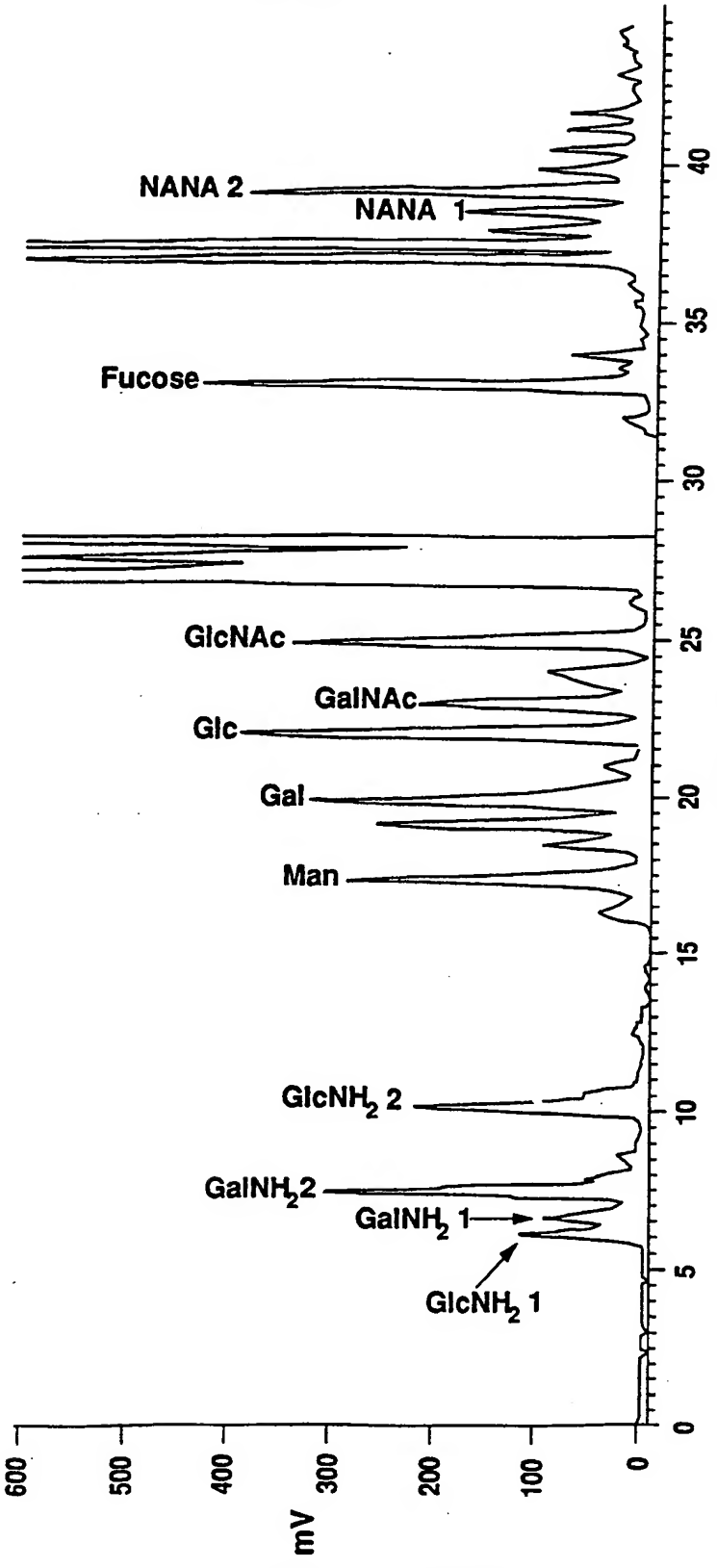

NANA 2
NANA 1
Fucose
GlcNAc
GalNAc
Glc
Gal
Man
GlcNH2 2
GalNH2 2
GalNH2 1
GlcNH2 1
0
5
10
15
20
25
30
35
40
MINUTES
600
500
400
300
200
100
0
mV

Fig. 16B

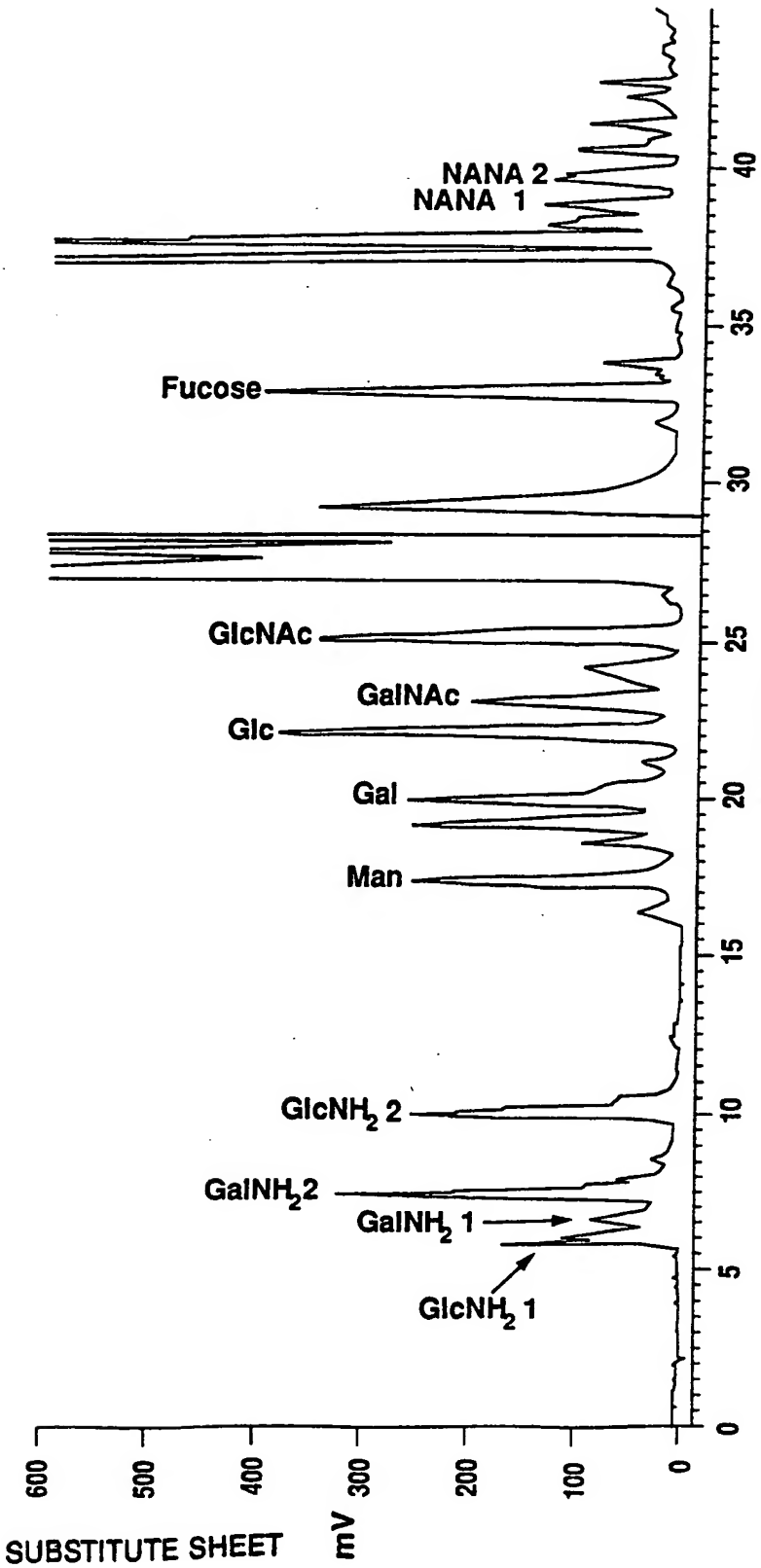

NANA 2
NANA 1
Fucose
GlcNAc
GalNAc
Glc
Gal
Man
GlcNH2 2
GalNH2 2
GalNH2 1
GlcNH2 1
0
5
10
15
20
25
30
35
40
MINUTES
600
500
400
300
200
100
0
mV

Fig. 16C

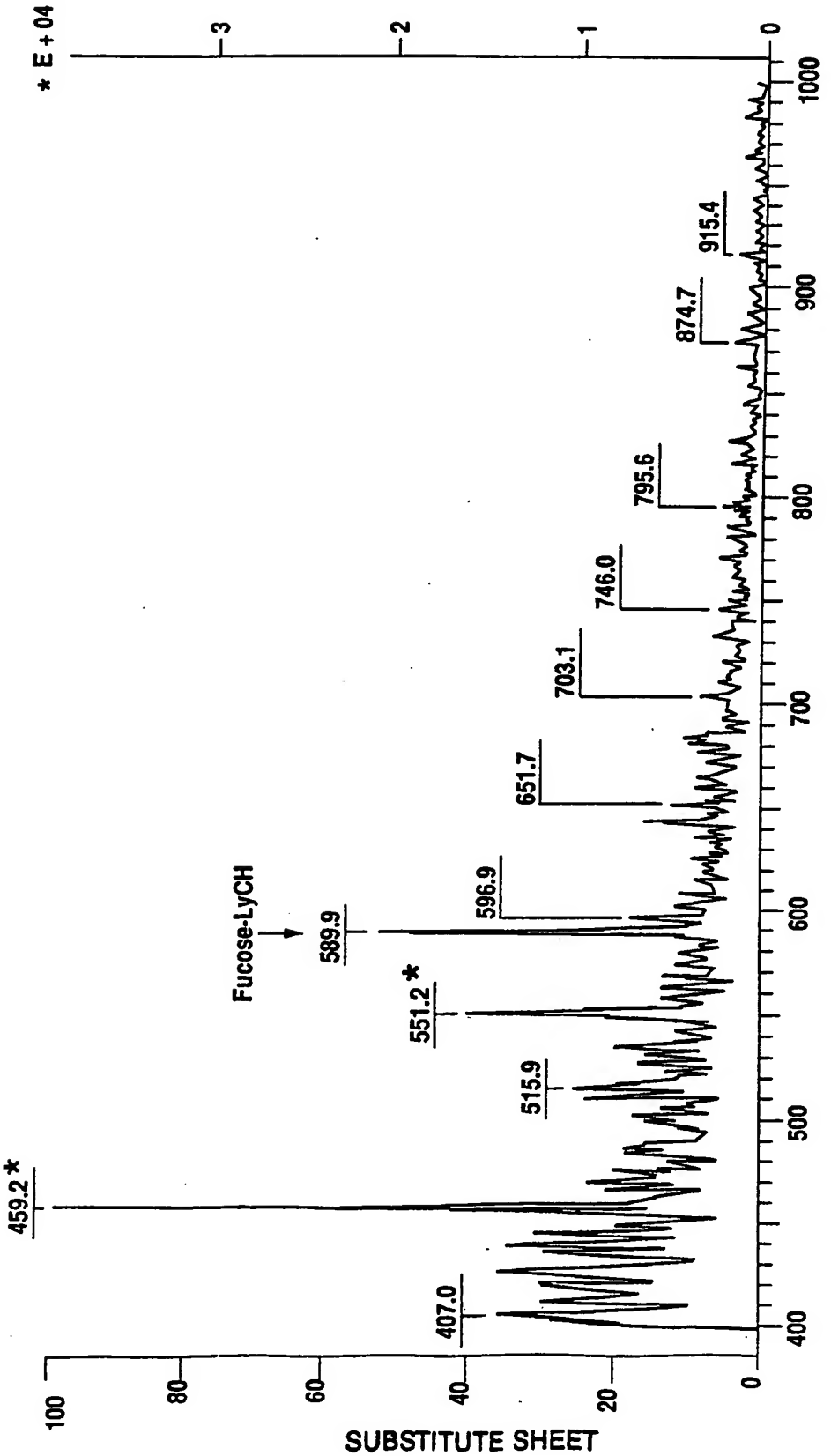

* E+04
3
2
1
0
100
80
60
40
20
0
400
500
600
700
800
900
1000
459.2*
407.0
515.9
551.2*
589.9
Fucose-LyCH
596.9
651.7
703.1
746.0
795.6
874.7
915.4

Fig. 17A

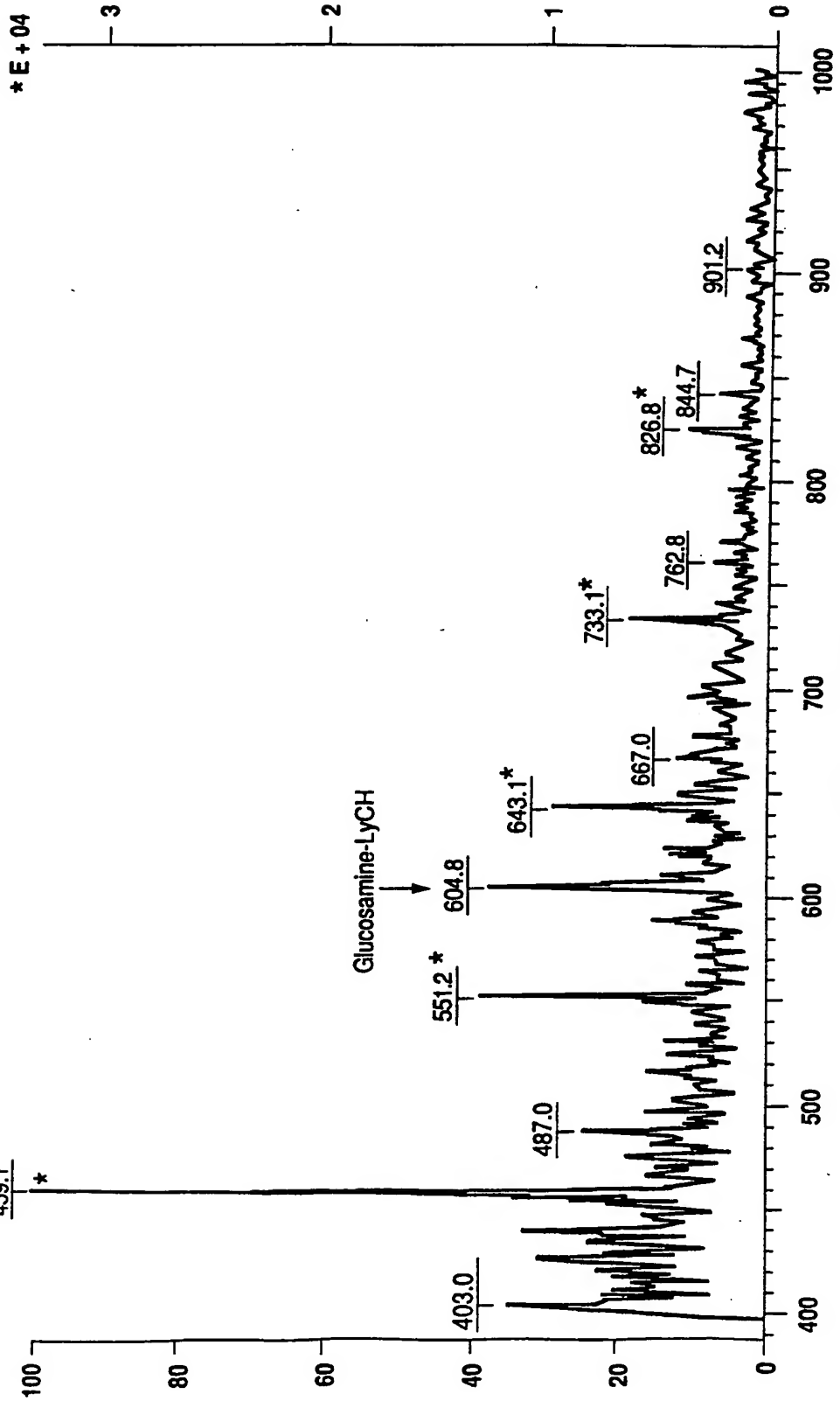

* E + 04
3
2
1
0
100
80
60
40
20
0
400
500
600
700
800
900
1000
459.1
*
403.0
487.0
551.2*
Glucosamine-LyCH
604.8
643.1*
667.0
733.1*
762.8
826.8*
844.7
901.2

Fig. 17B

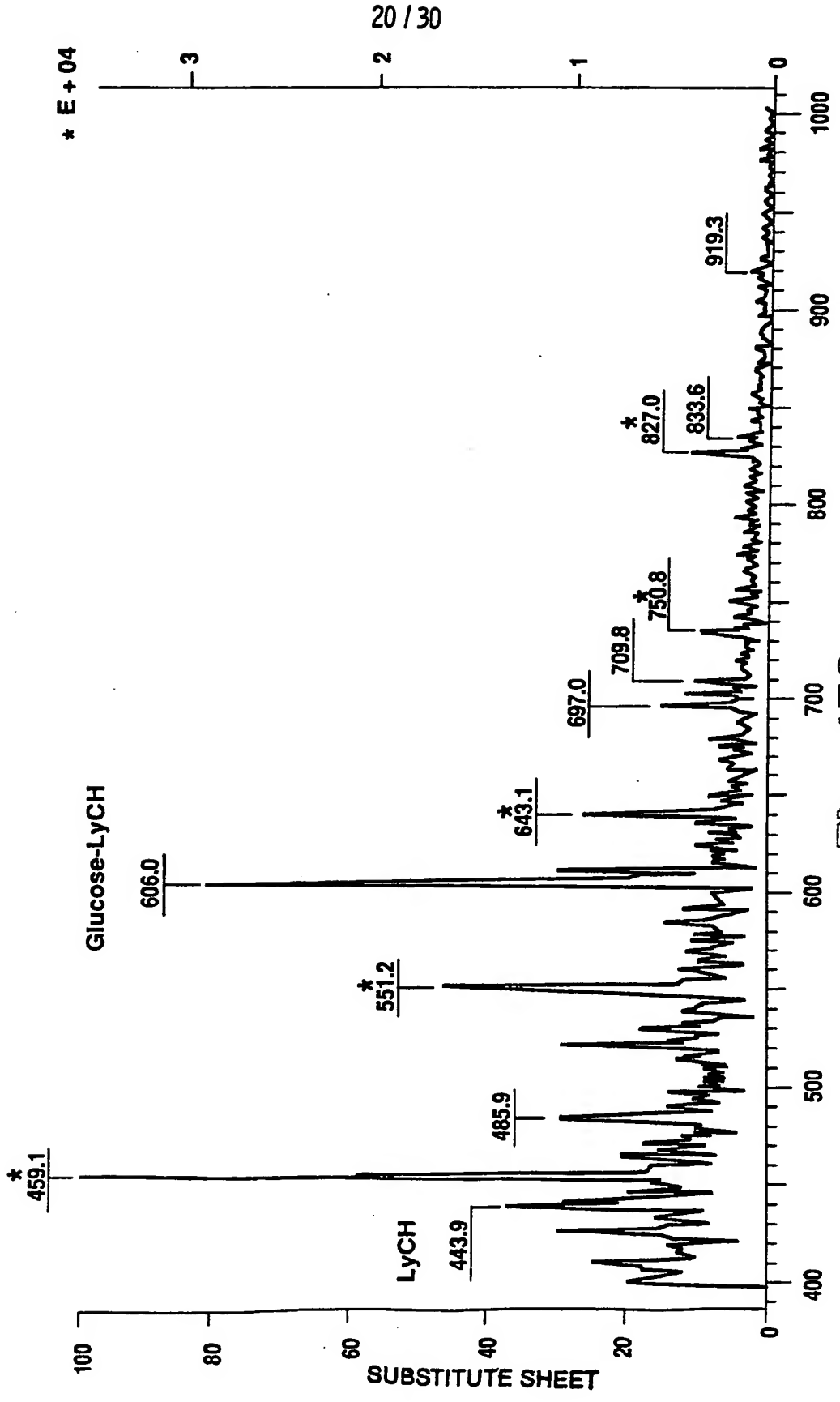

* E + 04
3
2
1
0
1000
900
800
700
600
500
400
919.3
833.6
827.0
750.8
709.8
697.0
643.1
606.0
551.2
485.9
459.1
443.9
Glucose-LyCH
LyCH
100
80
60
40
20
0
SUBSTITUTE SHEET

Fig. 17C

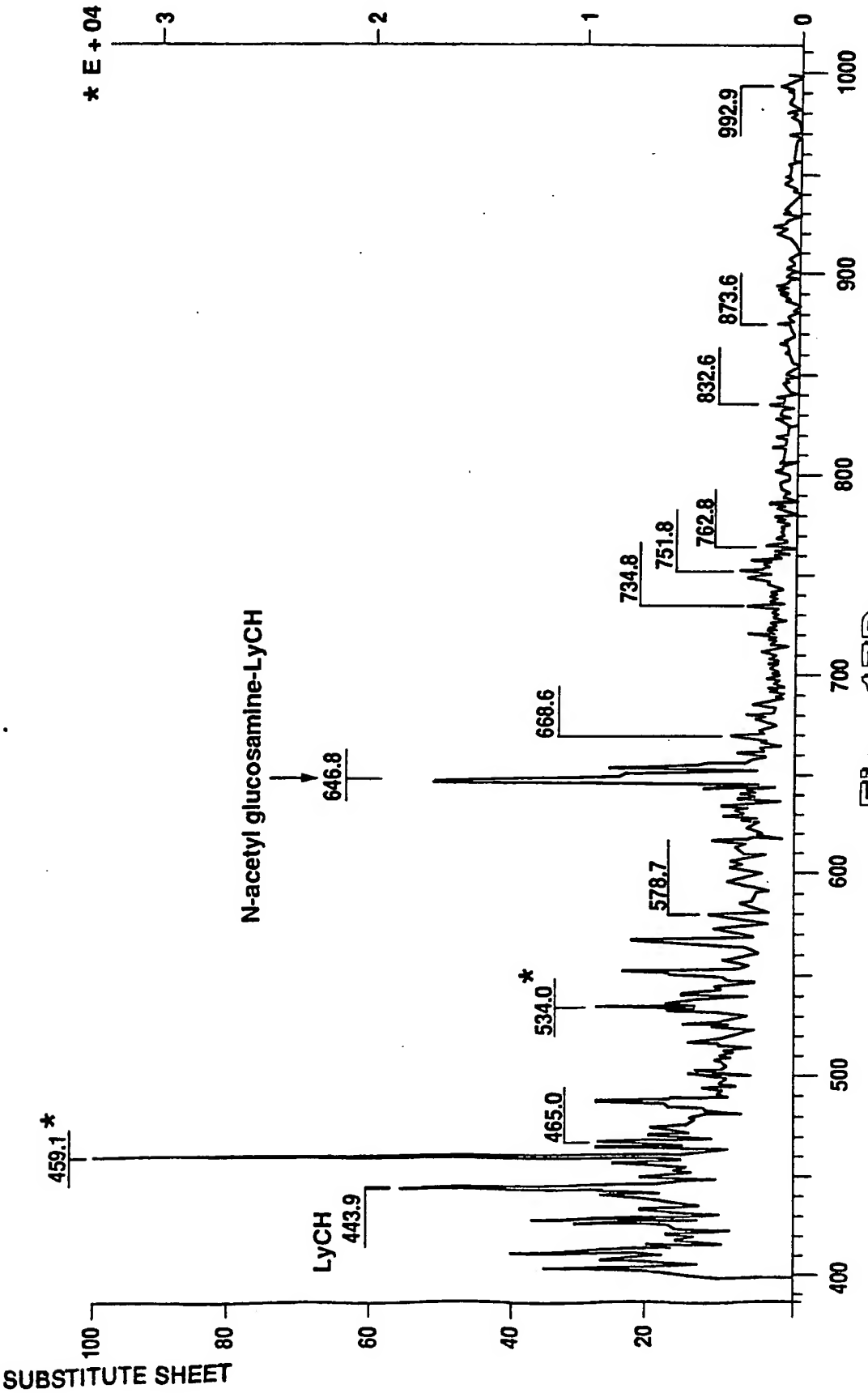

* E + 04
3
2
1
0
100
80
60
40
20
SUBSTITUTE SHEET
459.1 *
LyCH
443.9
465.0
534.0 *
578.7
N-acetyl glucosamine-LyCH
646.8
668.6
734.8
751.8
762.8
832.6
873.6
992.9
400
500
600
700
800
900
1000

Fig. 17D

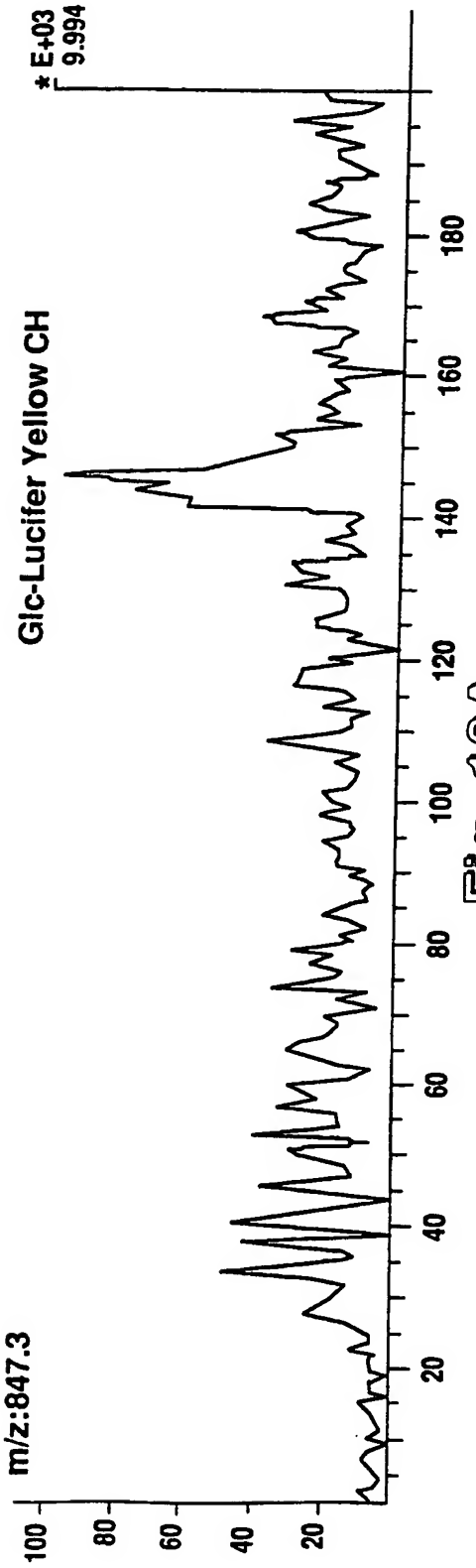


Fig. 18A

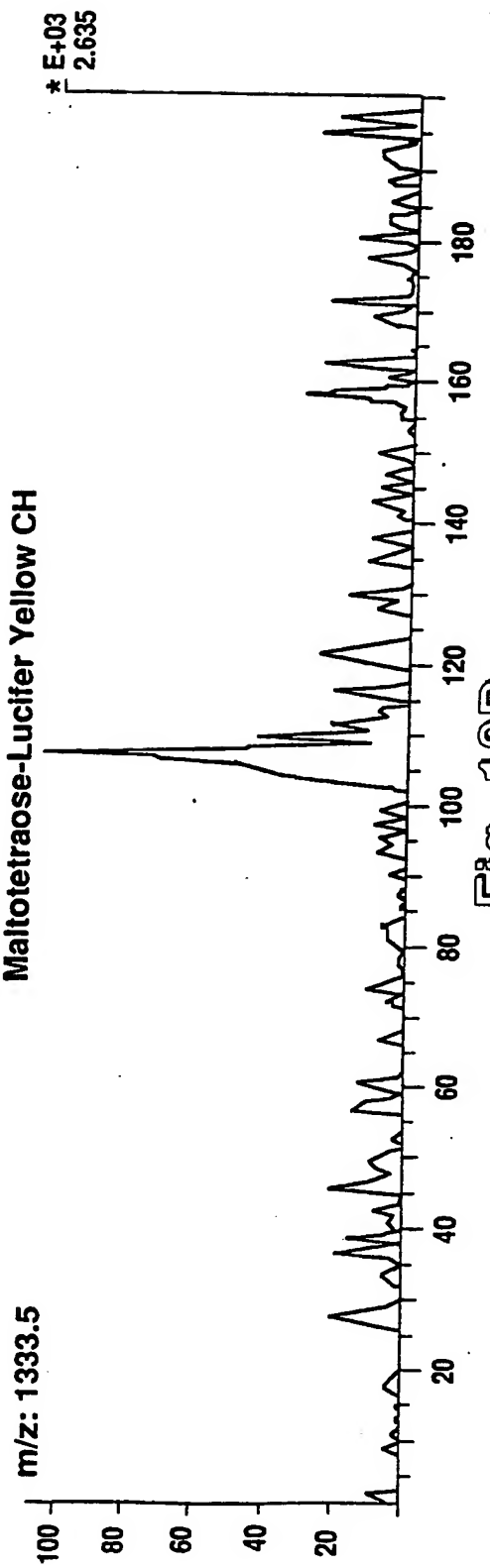


Fig. 18B

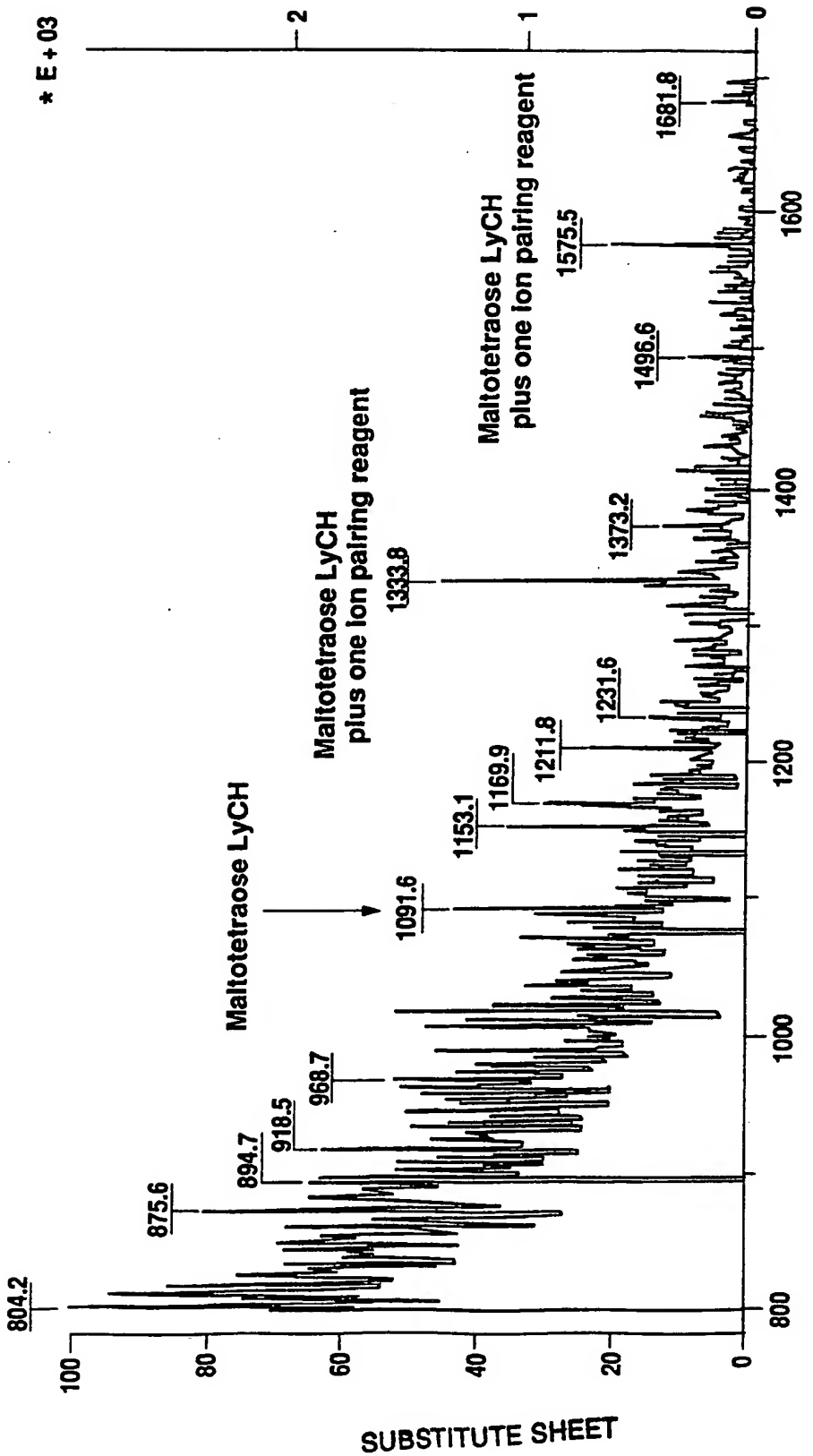

* E+03
2
1
0
Maltotetraose LyCH
Maltotetraose LyCH plus one ion pairing reagent
804.2
875.6
894.7
918.5
968.7
1091.6
1153.1
1169.9
1211.8
1231.6
1333.8
1373.2
1496.6
1575.5
1681.8
100
80
60
40
20
0
800
1000
1200
1400
1600

Fig. 19

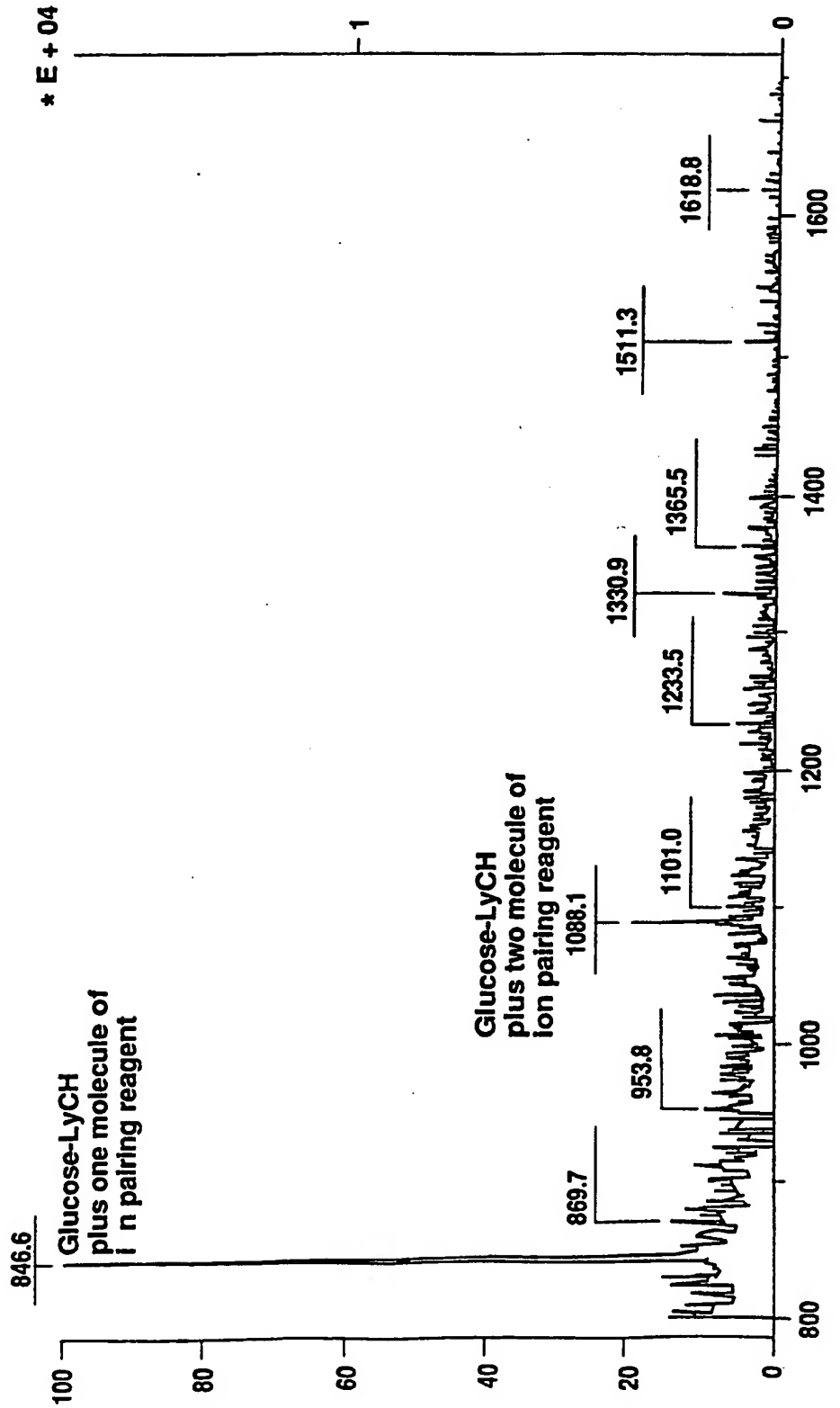

* E + 04
1
0
1618.8
1600
1511.3
1365.5
1400
1330.9
1233.5
1200
1101.0
1088.1
1000
953.8
869.7
800
846.6
Glucose-LyCH
plus one molecule of
i n pairing reagent
Glucose-LyCH
plus two molecule of
ion pairing reagent
100
80
60
40
20
0

Fig. 20

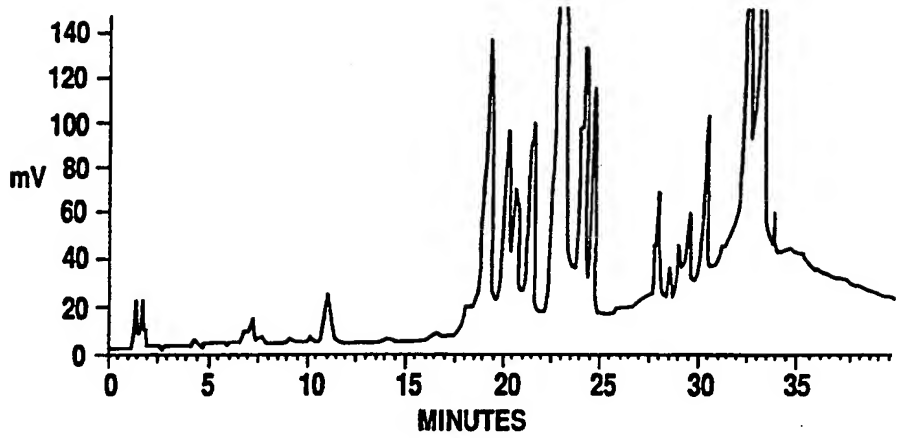


Fig. 21A-1

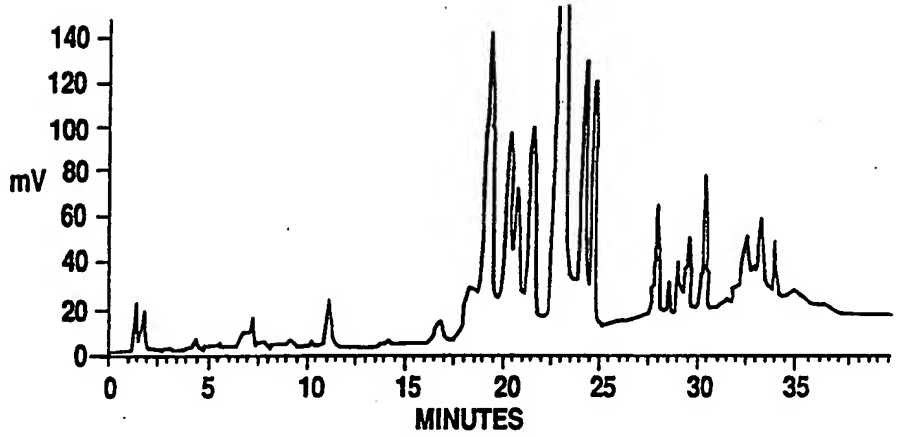


Fig. 21A-2

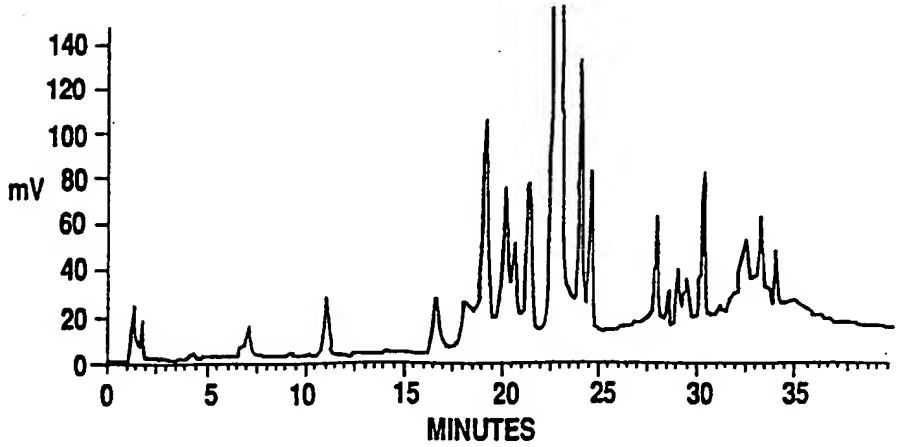


Fig. 21A-3

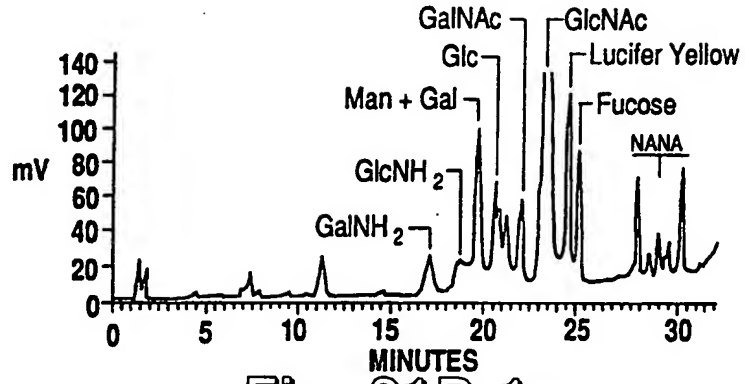


Fig. 21B-1

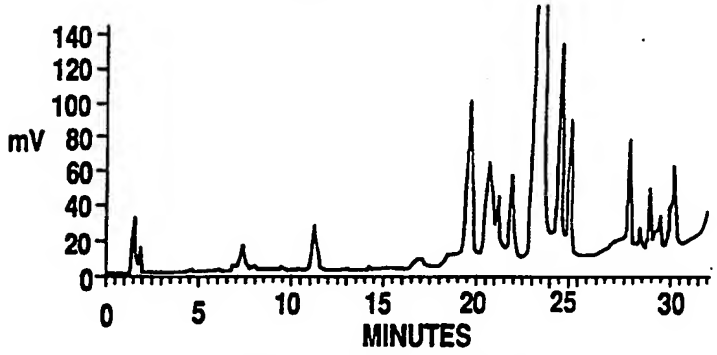


Fig. 21B-2

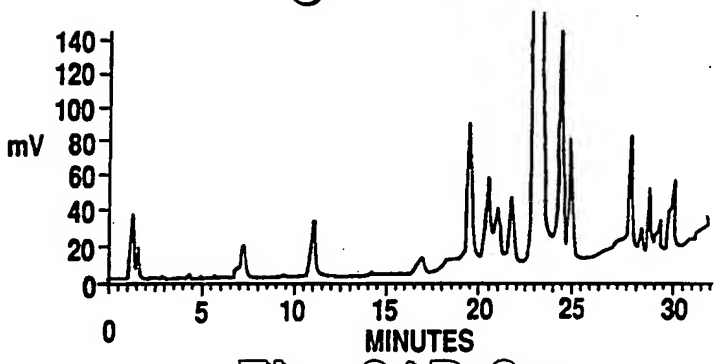


Fig. 21B-3

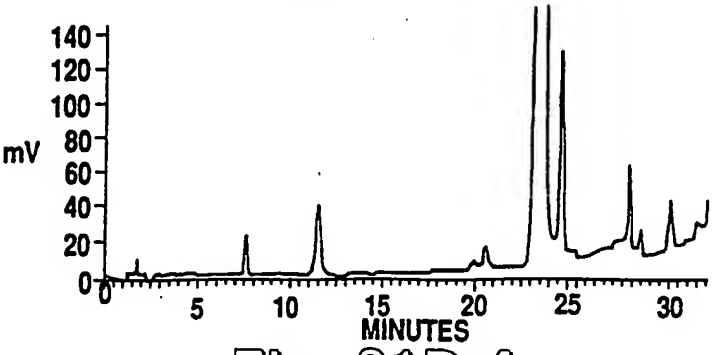


Fig. 21B-4

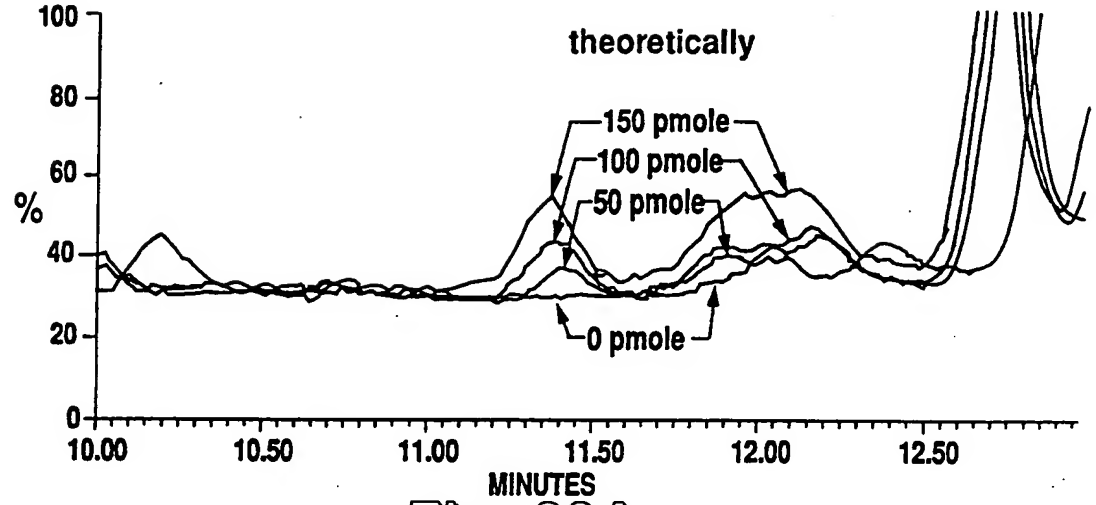


Fig. 22A

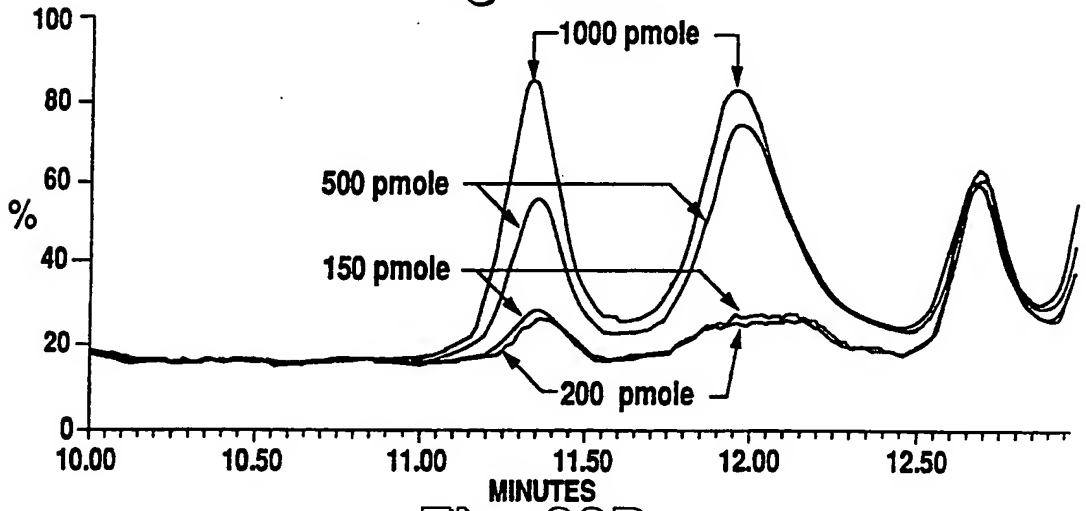


Fig. 22B

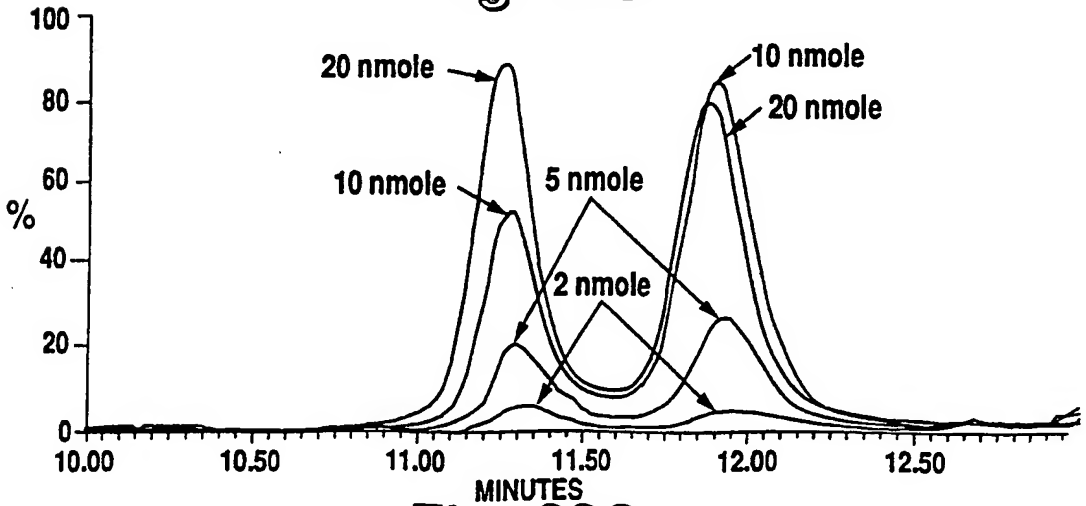


Fig. 22C

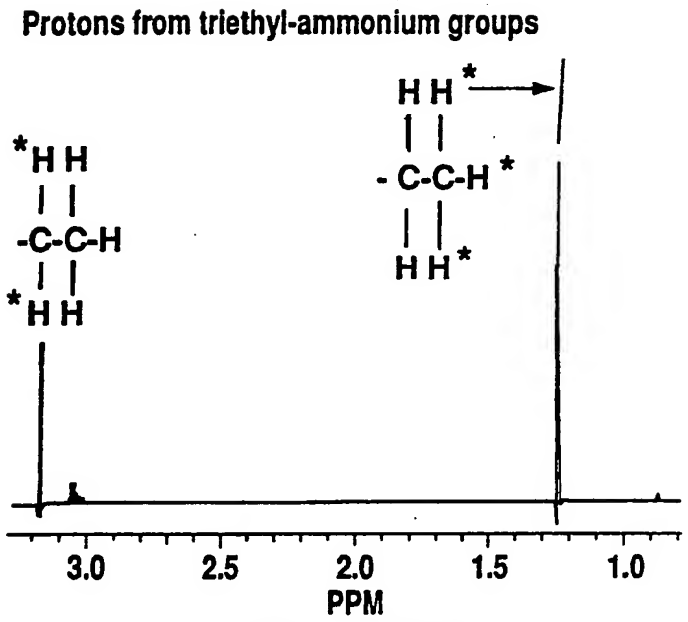


Fig. 23A

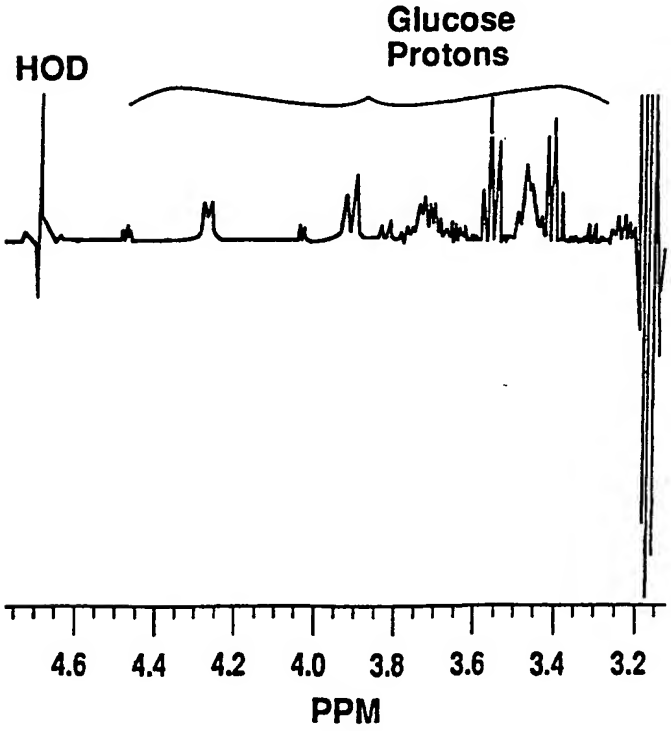


Fig. 23B

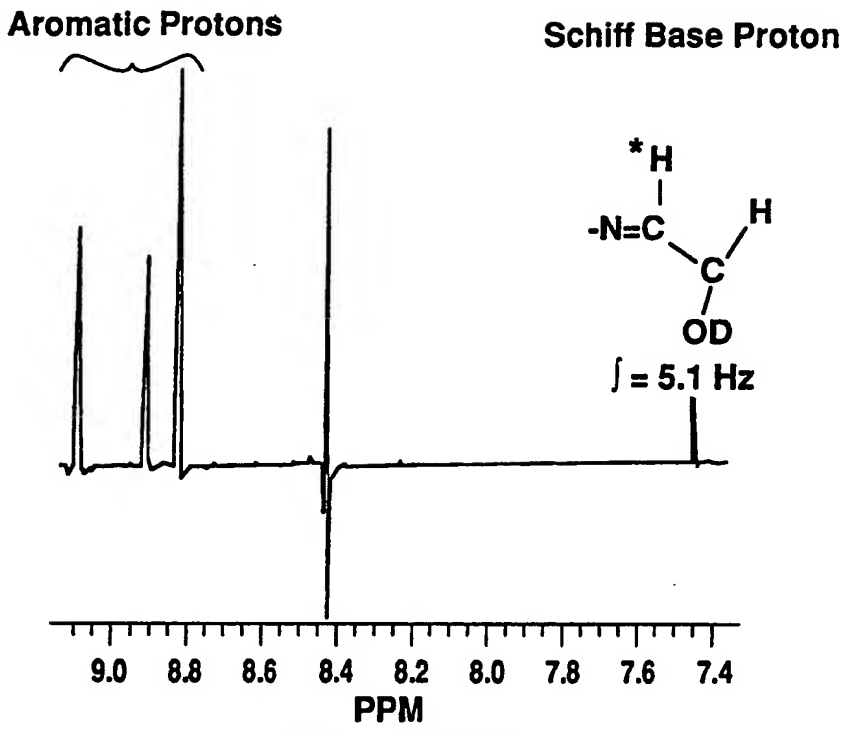


Fig. 23C

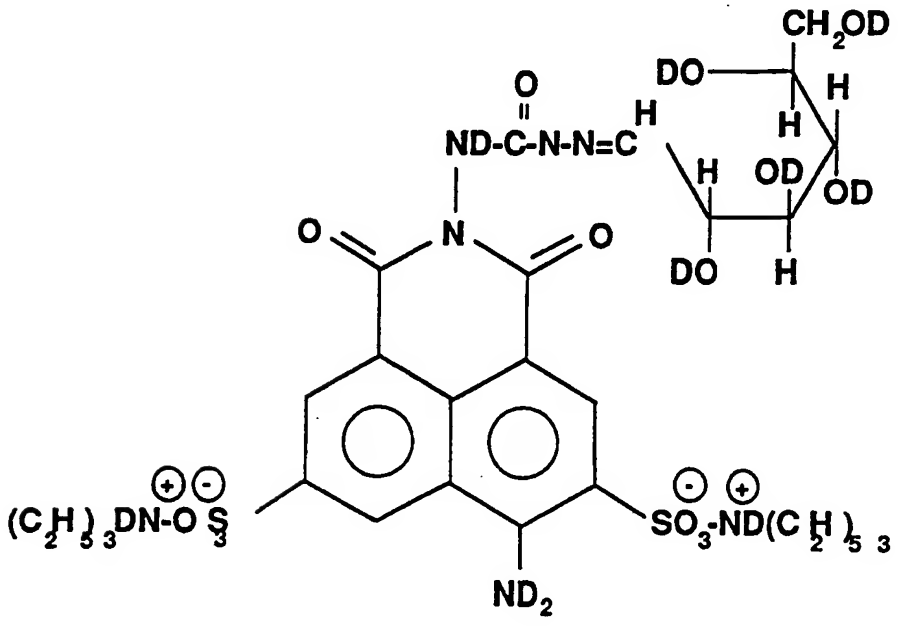


Fig. 23D

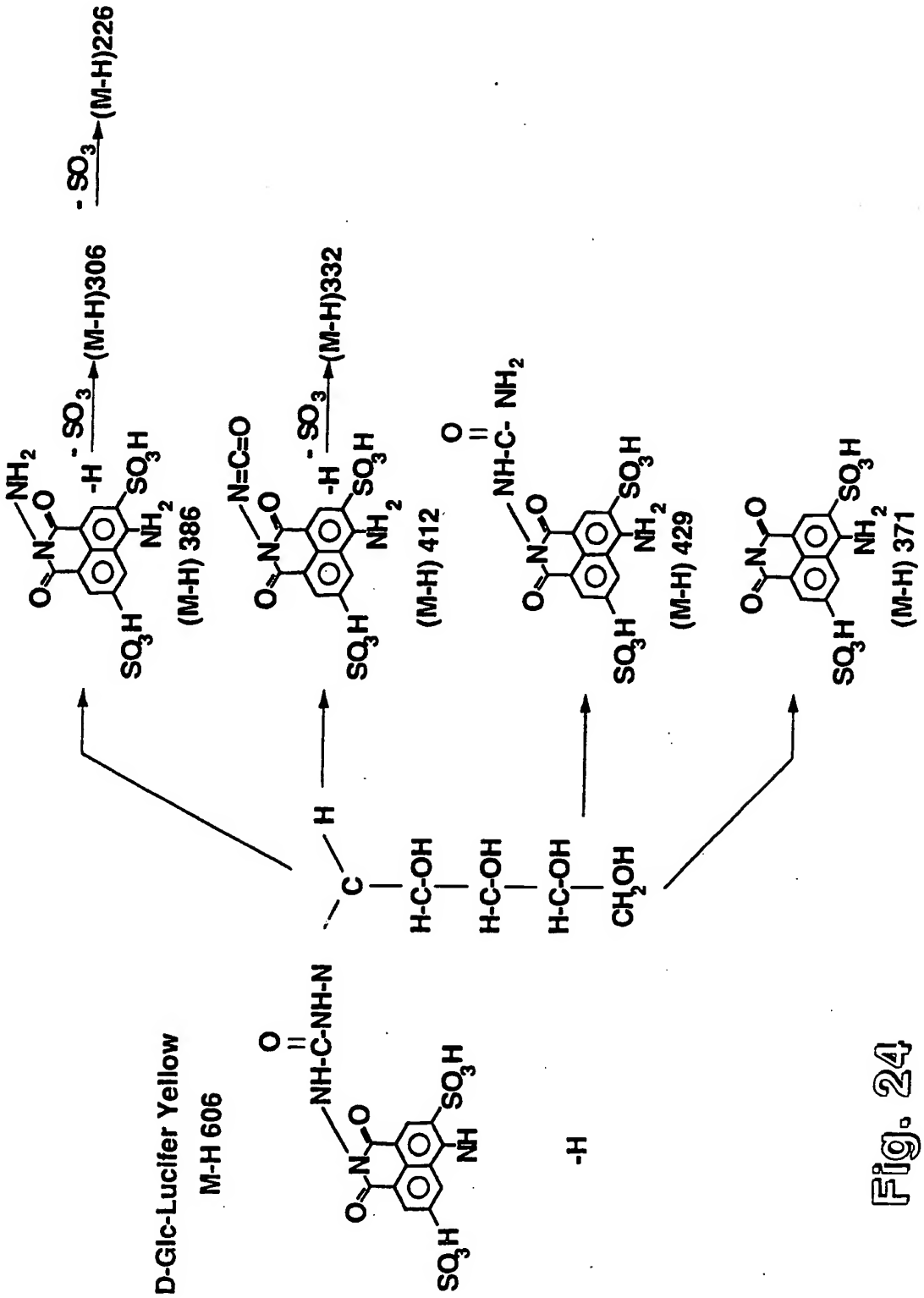

D-Glc-Lucifer Yellow
M-H 606
-H
(M-H) 386
(M-H) 412
(M-H) 429
(M-H) 371
(M-H)306
(M-H)332
(M-H)226

Fig. 24

TERNATIONAL SEARCH REPORT

International Applica..on PCT/US 91/03824

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 H 15/26 G 01 N 33/52 G 01 N 33/68
G 01 N 33/66

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	G 01 N C 07 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	Biochemistry, vol. 24, no. 2, 1985, Easton, Pa, US; J.A. Lee et al.: "Labeling of the glycoprotein subunit of (Na,K)ATPase with fluorescent probes", pages 322-330 ---	1-23
Y	Analytical Chemistry, vol. 59, no. 9, 1 May 1987, Columbus, Ohio, US; J.K. Lin et al.: "Synthesis of dabsylhydrazine and its use in the chromatographic determination of monosaccharides by thin-layer and high-performance liquid chromatography", pages 1320-1326 ---	1-23
Y	Journal of Chromatography, vol. 244, no. 2, 1982, Elsevier Scientific Publ. Co. Amsterdam, NL; M. Takeda et al.: "Fluorescence high-performance liquid chromatography of reducing sugars using Dns-hydrazine as a pre-labelling reagent", pages 347-355 --- -/-	1-23

[10] Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07-10-1991	23. 10. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS M. Peis

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Chemical Abstracts, vol. 99, no. 3, 18 July 1983, Columbus, Ohio, US; S. Spiegel et al.: "Fluorescence labeling of cell surface glycoconjugates with Lucifer Yellow CH.", see page 322, abstract 19086y, & Biochem. Biophys. Res. Commun. 1983, 112(3), 872-877	
A	Journal of the American Chemical Society, vol. 103, no. 25, 1981, American Chemical Society, Easton, Pa, US; W.W. Stewart.: "Synthesis of 3,6disulfonated 4-aminophthalimides", pages 7615-7620	